



Do mixed infections matter? Assessing virulence of mixed-clone infections in experimental human and murine malaria



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ABSTRACT

Background: Malaria parasites within an individual infection often consist of multiple strains (clonal populations) of a single species, which have the potential to interact both with one another, and with the host immune system. Several effects of these interactions have been measured in different parasite systems including competition and mutualism; however, direct observation of these effects in human malaria has been limited by sampling complexities and inherent ethical limitations.

Methods: Using multiple complementary epidemiological models, we propose a suite of analyses to more fully utilize data from challenge experiments, and re-examine historical human challenge studies with mixed-strain *Plasmodium vivax* inocula. We then compare these results with murine model systems using mixed-strain *Plasmodium yoelii* or *Plasmodium chabaudi*, to explore the utility of these methods in fully utilizing these data, including the first quantitative estimates of effect sizes for mixed-strain parasitemia. These models also provide a method to assess consistency within these animal model systems.

Results: We find that amongst a limited set of *P. vivax* (incubation time) and *P. yoelii* infections (time-to-mortality), survival times at a study population-level are intermediate between each single-clone infection, and are not dominated by the more virulent clone; in *P. vivax* relapses, mixed clone infections also show intermediate survival curves. In these infections, the results strongly suggest that highly virulent clones have their virulence attenuated by the presence of less-virulent clones. The analysis of multiple experiments with *P. chabaudi* suggests greater nuances in the interactions between strains, and that mortality and time-to-event in mixed-strain infections are both indistinguishable from single infections with the more virulent strain.

Conclusions: These divergent dynamics support earlier work that suggested drivers of virulence may differ in fundamental ways between malaria species that are reticulocyte-specific and those that readily infect all red blood cell stages which should be studied in greater detail. The effect sizes (magnitude of biological effects) from these analyses are significant, and suggest the potential for important gains in malaria control by greater incorporation of evolutionary epidemiology theory. Moreover, we suggest that using these epidemiological models may generally allow fuller use of data from experimentally challenging animal model experiments.

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1. Introduction

Malaria is a major contributor to morbidity and mortality globally, with an estimated 198 million cases (95% CI: 124 to 283) and 584,000 deaths (95% CI: 367,000 to 755,000) in 2013 (World Health Organization, 2014). There are five species that generally infect humans, and the interactions between different species within a single infection can have important clinical implications (Zimmerman et al., 2004). Moreover, infections within an individual may be composed of

multiple strains (clonal populations) of a single species, which may interact both with one another and with the host immune system (Alizon et al., 2011).

The impacts of these interactive parasite populations have been explored through multiple lines of research using two complementary approaches: mathematical/statistical models, and experimental animal systems. The interactions between parasite populations may lead to parasite competition or to parasite mutualism; to the evolution of virulence and drug susceptibility; and may facilitate genetic exchange within infections (Balmer and Tanner, 2011). Moreover, these assemblages of hosts and multiple parasites form an evolutionary community, and the incorporation of ecological and evolutionary theory (Alizon et al., 2009; Rigaud et al., 2010), has led to the development of

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evolutionary epidemiology as a field in itself (Restif, 2009). However the impacts of these parasite interactions on the host are highly variable, and observed virulence in mixed-strain infections ranges along a continuum from being greater than the more virulent parasite; less than the least virulent strain; or any intermediate virulence between these extremes (Alizon et al., 2013).

In most epidemiological settings, the majority of individual *Plasmodium vivax* and *Plasmodium falciparum* infections are composed of multiple strains e.g., (Henry-Halldin et al., 2011; Juliano et al., 2010), and these infections may represent an underutilized tool to explore complex transmission dynamics (Bordes and Morand, 2009; de Araujo et al., 2012). However, the inherent difficulties of data capture across multiple spatial and temporal scales, and practical limitations in data collection from parasitized hosts in natural environments have led to suggestions for increased multidisciplinary research including the use of more diverse methodologies to triangulate findings across different specializations (Restif et al., 2012).

This work aims to address three specific questions: i) how can a broader range of analytical approaches contribute to what is known about mixed-strain infections with *Plasmodium* and within-host dynamics?; ii) are results from the limited human challenge studies consistent with what is known from murine model systems?; and iii) do these results support any specific drivers of virulence?

1.1. Strain theory and virulence

The concept of parasite ‘strains’ is pervasive throughout the medical and malaria literature, however, no consensus has emerged on what these clinical isolates represent (Balmer and Tanner, 2011; McKenzie et al., 2008); in general they refer to clonal or at least closely-related populations.

While results have not been entirely consistent, themes have emerged that appear to be general across parasite and host species (Alizon et al., 2013). Observational data suggest that clones may manifest with diverse clinical impacts, including differences in virulence, clinical severity, transmissibility, and both the number and spacing of hypnozoite-derived relapses in *P. vivax* infections (reviewed in McKenzie et al., 2008). Recent molecular and genetic approaches have added to this knowledge base by characterizing both inter- and intra-host infections; these studies suggest that while many strains/isolates represent a diversity of clonal populations, they generally produce stable clinical and immunological responses (McKenzie et al., 2008). Moreover, consideration of virulence metrics *across the entire duration of infection* is critical to understand the pressures that drive strain selection (Barclay et al., 2014).

Virulence in malaria infections is a composite outcome of at least three processes: specific hematological impacts; host immune response to toxins (including hemozoin and other parasitic debris); and cyto-adhesion in some parasite species including *P. falciparum* (reviewed in Mackinnon and Read, 2004; Wassmer et al., 2015). Assessments of the relative impact of these divergent processes have been the subject of many experimental studies, and new modeling approaches have been developed to disentangle the specific physiological processes behind the composite endpoint of ‘virulence’ (Metcalf et al., 2011). These and related studies suggest that the relative contribution of each of these facets of observed virulence can vary dramatically during the time course of an infection in a strain-specific manner (Metcalf et al., 2012).

As noted by Alizon and coworkers, theoretical models almost universally define virulence as an increase in host mortality, but in the broader parasitology literature virulence is considered to be any harm caused by a parasite that negatively impacts host fitness (Alizon et al., 2013). While a range of definitions have appeared (Casadevall and Pirofski, 2001; Poulin and Combes, 1999), herein we consider it as ‘parasite populations that maximally exploit host resources’ to capture the

range of endpoints examined in this study, with a focus on direct parasite-induced mortality.

1.2. Previous analytical strategies

In general, previous analyses of murine systems have focused on pooled hematological or parasitological outcomes as proxies for virulence (comparing means or geometric means from all surviving animals) e.g., (Bell et al., 2006), but full multivariate analyses of outcomes or explicit consideration of times-to-events have received very limited attention. In the original analysis of Bell et al., differences between single- and mixed-strain infections were not examined; virulence was assessed by maximum anemia and parasitemia and showed broad trends, but with complex relationships between the two metrics [e.g., Fig. 1 in (Bell et al., 2006)].

Moreover, while time itself is a critical component of virulence (Day, 2002), analyses have generally focused on analysis at several (potentially arbitrary) time points. In many cases, censoring of subjects has not been considered – that is, animals are simply removed from analysis; beyond a potential for introducing biases, this greatly reduces the power of the analysis (Rothman et al., 2008). Finally, most parasitological studies report only p-values to evaluate significance, as is the norm in ecology; however, these do not allow consideration of the effect sizes. That is, statistically significant effects may not be clinically or biologically important- or as well-phrased by others, “Identifying biological importance is what all biologists are ultimately aiming for, not the identification of statistical significance.” (Nakagawa and Cuthill, 2007). Although one prior study measured the statistical significance of virulence (Taylor et al., 1998), we are unaware of any prior effect size estimates.

2. Materials and methods

The human study data (Dataset I; Table 1) in this analysis was obtained during a series of rigorous challenge experiments in prison volunteers during the 1940s–50s in an antimalarial drug development program; all case-patients were malaria-naïve white males. Infections were via mosquito challenge, there was complete follow-up, and patients were protected from superinfection due to the institutional nature of this population. The study population (Table 1) includes a less-virulent historical strain from North America and a highly virulent clone from Papua New Guinea (Collins, 2013; Ungureanu et al., 1976). Full details for human challenge infections including search criteria and primary references were previously published (Lover and Coker, 2013); mixed-strain challenges can be found in (Cooper et al., 1950).

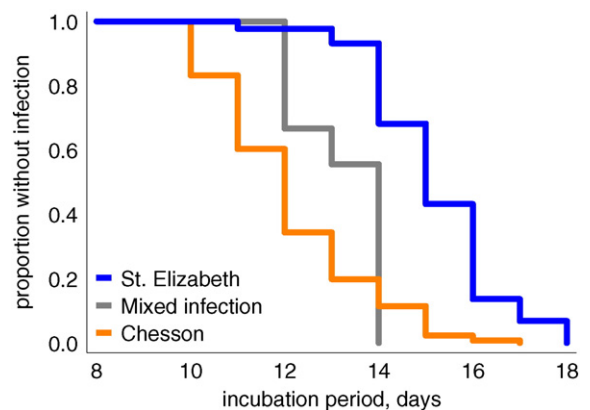


Fig. 1. Kaplan–Meier curves comparing incubation periods in single strains and mixed-strain infections in human challenge experiments with *Plasmodium vivax* (Dataset I; N = 184).

Table 1
Study population for historical human challenge experiments with *Plasmodium vivax* (Dataset I).

End point	Treatment	n	% of total
Incubation period	Mixed	9	4.9
	Chesson	131	71.2
	St. Elizabeth	44	23.9
	Total	184	100.0
Time-to-first relapse	Mixed	9	13.6
	Chesson	19	28.8
	St. Elizabeth	38	57.6
	Total	66	100.0

Relapse times are presented as time from mosquito inoculation; all single-strain infections had symptomatic-only treatment with quinine; and all mixed-strain inoculations had chloroquine exposure after the primary attack.

Data for *P. yoelii* infections (Dataset II; Table 2) were extracted from (Hargreaves et al., 1975); although the authors refer to these parasites as *Plasmodium berghei yoelii*, a later revision elevated these to the species-level as *P. yoelii* (Killick-Kendrick, 1974; Perkins et al., 2007). CF1 mice were inoculated with 10^6 parasitized RBCs, and 2×10^6 in the mixed infections. The mild strain of '17X' was obtained from a wild thicket rat; the virulent form was obtained after serial passages in rodents and *Anopheles stephensi* (Hargreaves et al., 1975).

Data for the first set of *P. chabaudi* murine challenge experiments (Dataset III; Table 3) were taken from (Snounou et al., 1992). Male CBA/ca mice were inoculated with 10^4 parasitized erythrocytes; mixed infections were inoculated with 1×10^4 of each strain. Data for the later murine challenges with *P. chabaudi* (Dataset IV; Table 4) are from the work of Bell et al. and are available from the Drydad data repository (Bell et al., 2014; de Roode et al., 2005). In these infections, C57BL/6J inbred female mice were inoculated with 10^6 parasites; mixed infections received a total dose of 2×10^6 ; prior work suggests no impact of this 2-fold difference (Bell et al., 2006). The strains were chosen specifically to mimic the potential diversity within wild parasite populations. Our analysis was focused on the initial acute phase of primary infections before specific immunological responses occur; therefore surviving mice were censored one day past the last recorded fatality (on day 15).

2.1. Ethics

This study analyzes fully de-identified, secondary data published in the open literature and in the public domain; no ethics review was required. There has been prior detailed discussion about ethical aspects of the analysis of data from the prison volunteers in these studies (Harcourt, 2011; Weijer, 1999).

2.2. Methods

Preliminary analyses of the relationship between parasite strains and times-to-event used non-parametric analyses (Kaplan–Meier curves); differences were compared using the Peto–Peto test due to differences in censoring and non-proportional hazards (Klein and Kleinbaum, 2005; Lachin, 2011). Following this, multivariate analysis (survival and/or log binomial models for time-to-event or mortality)

Table 2
Study population for murine challenge experiments with *Plasmodium yoelii* (Dataset II).

Treatment	n	% of total	Mortality % (95% CI)
Mixed	6	33.3	100 (54.1–100)
'17X mild'	6	33.3	50 (11.8–88.2)
'17X virulent'	6	33.3	100 (54.1–100)
Total	18	100.0	–

Table 3
Study population for murine challenge experiments with *Plasmodium chabaudi* (Dataset III) (Snounou et al., 1992).

Treatment	n	% of total	Mortality % (95% CI)
AS	10	10	20 (2.5–55.6)
CB	10	10	80 (44.4–97.5)
DS	10	10	60 (26.2–87.8)
AS + CB	10	10	100 (69.2–100)
CB + DS	10	10	100 (69.2–100)
AS + CB	10	10	40 (12.2–73.8)
Total	60	100.0	–

was used to estimate the magnitude of observed effects. Parametric and semi-parametric survival models were explored in the analysis of these experimental data; extensive issues were observed with lack-of-fit, non-convergence due to collinear strains, crossing survival curves, or complete separation. Moreover, standard survival models are not applicable in the presence of crossing survival curves (Li et al., 2015).

Due to these analytical issues, a set of complementary analyses was performed to allow all the studies to be analyzed within a consistent analytical framework. First, pseudovalues were computed and used to compare differences in restricted mean survival times (Parner and Andersen, 2010; Andersen and Perme, 2010); these methods specifically assess differences when hazard ratios may be inaccurate; the restricted mean survival times (RMSTs) were calculated at just before the last observed event time (symbolized as t^*) (Royston and Parmar, 2011). Secondly, the incidence rate ratios (that is, the ratio of total number of events per follow-up time per strain) were compared.

Finally, where possible, full multivariate methods were used to assess the magnitude (effect size) of strain differences. For the study of *P. yoelii* mortality (Dataset II), flexible parametric survival models were used; these are comprehensive alternatives to standard Cox survival models which allow greater flexibility in both model-fitting and prediction (Royston and Lambert, 2011; Royston and Parmar, 2002).

For the first study of *P. chabaudi* mortality (Dataset III) regressions for a binomial outcome (mortality) were used to quantify the differences in mortality between strains as times-to-event was not reported. Due to very high mortality, logistic regression models performed poorly. Alternative models (log-binomial and so-called robust Poisson models) were used and provided comparable estimates; the Poisson models are reported (Chen et al., 2014; Zou, 2004). For the second study of *P. chabaudi* mortality (Dataset IV) regressions for a mortality outcome also were used to quantify the differences in mortality between strains. Both model parameterizations had convergence issues, so the 'COPY' was used to facilitate model convergence (Cummings, 2009; Petersen and Deddens, 2006).

Table 4
Study population for murine challenge experiments with *Plasmodium chabaudi* (Dataset IV) (Bell et al., 2006).

Treatment	n	% of total	Mortality % (95% CI)
1. AS	10	10	0 (0–30.8)
2. AJ	10	10	70 (34.8–93.3)
3. AT	10	10	30 (6.7–65.2)
4. CB	5	5	60 (14.7–94.7)
5. AS + AJ	10	10	30 (6.7–65.2)
6. AS + AT	10	10	50 (18.7–81.3)
7. AS + CB	5	5	40 (5.3–85.3)
8. AJ + AT	10	10	50 (18.7–81.3)
9. AJ + CB	5	5	100 (47.8–100)
10. AT + CB	5	5	80 (28.4–99.5)
11. CW	5	5	0 (0–52.2)
12. AS + CW	5	5	20 (0.51–71.6)
13. AJ + CW	5	5	20 (0.51–71.6)
14. AT + CW	5	5	40 (5.3–85.3)
Total	100	100.0	–

For all models parsimony was assessed using Akaike and Bayesian information criteria (AIC/BIC); residuals and standard goodness-of-fit tests were used to assess adequacy of the robust Poisson and restricted mean survival models; the fit of the flexible parametric survival model was judged using overlaid comparisons with Kaplan–Meier curves. ‘Robust’ (Huber–White) error structure was used in all models to address the non-independence of subjects within study arms (Williams, 2000). Models were assessed for proportional hazard violations using Schoenfeld residuals.

All analyses were performed using Stata 13.1 (College Station, TX, USA) and all tests were two-tailed, with $\alpha = 0.05$.

3. Results

3.1. *P. vivax* infections – incubation period

The results from Kaplan–Meier analysis of incubation periods (time from mosquito exposure to febrile illness; Dataset I) are shown in Fig. 1 and Table 5. Patients infected with the Chesson strain had the shortest incubation periods, with intermediate (and crossing) curves from mixed infections, followed by the St. Elizabeth strain infections. The arithmetic median incubation periods were: Chesson 12 days (95% CI: 12 to 12), mixed infection 14 (12 to 14), and St. Elizabeth 15 (95% CI: 15 to 16), and the differences in restricted mean survival time (RMST) are all significant (mixed infection vs. St. Elizabeth; and Chesson vs. St. Elizabeth, both $p < 0.001$; Chesson vs. mixed, $p = 0.001$).

Comparisons between incidence rate ratios are consistent with the Kaplan–Meier analysis: infections with the St. Elizabeth strain had an IRR of 0.80 (95% CI: 0.77–0.83; $p < 0.001$) relative to the more virulent Chesson strain. Relative to mixed inoculations, Chesson infections had an incidence rate ratio (IRR) of 1.1 (1.03–1.15; $p < 0.001$), with St. Elizabeth having an IRR of $= 0.87$ (0.82–0.91; $p < 0.001$). That is, over the follow-up period, case-patients infected with Chesson parasites had a ~10% faster rate, and those infected with St. Elizabeth parasites a ~13% slower rate of events, relative to case-patients infected with mixed-strain parasites.

3.2. *P. vivax* infections – relapses

Kaplan–Meier plots for time-to-first relapse are shown in Fig. 2. Rapid times to relapse are apparent in infections with the Chesson strain, followed by an intermediate curve from mixed inoculations, with St. Elizabeth relapses having the slowest times-to-relapse, spaced out over ~10 months. The median uncensored relapse times are: Chesson, 6.3 weeks (95% CI: 5.6 to 7.1); mixed, 11.4 weeks (9.2 to 38.5); and St. Elizabeth, 41.7 weeks (39.5 to 43.0).

Comparison of the incidence rates for relapses (Table 6) suggests that relative to the Chesson strain, infections with the St. Elizabeth strain have an IRR of 0.23 (95% CI: 0.13–0.41, $p < 0.001$). In comparison to mixed strain infections, infections with the St. Elizabeth strain had an IRR of 0.44 (0.27–0.70, $p = 0.001$), while comparison with the Chesson infections do not achieve significance, with an IRR of 1.9 (0.92–3.9; $p = 0.081$). That is, in single strain infections with St. Elizabeth parasites, there is ~2.3 fold, and ~4.3 fold, slower rate of relapse relative to mixed infections and to Chesson strain infections, respectively, while mixed infections do not show a significant difference from infections with Chesson parasites. Comparison of the RMSTs at $t^* = 45$ weeks

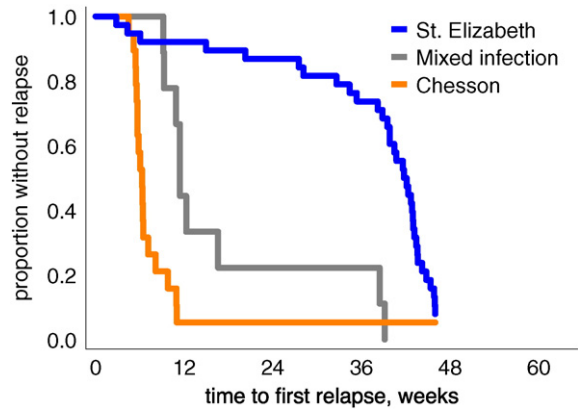


Fig. 2. Kaplan–Meier curves comparing time-to-first relapse in single strains and mixed-strain infections in human challenge experiments with *Plasmodium vivax* (Dataset I; N = 66).

are significant for both St. Elizabeth vs. Chesson ($p < 0.001$) and vs. mixed infection ($p < 0.001$); and the Chesson infections show only a marginally significant difference from mixed strain infections ($p = 0.042$).

3.3. *P. yoelii* infections

The results from survival analysis of mortality outcomes in experimental murine *P. yoelii* infections are shown in Fig. 3. There are clearly disenable and statistically significant differences in the time-to-mortality between both of the single strains and mixed infections, and between the two single-strain infections. In Kaplan–Meier analysis, the Peto–Peto log-rank test indicates significant differences between the strains (‘17X-mild’ vs. ‘17X-virulent’, $p = 0.0010$; ‘17X-virulent’ vs. mixed, $p = 0.0103$; ‘17X-mild’ vs. mixed, $p = 0.0034$); comparison of the RMST differences (Table 7) at $t^* = 22$ days shows comparable p-values (‘17X-mild’ vs. ‘17X-virulent’, $p < 0.001$; ‘17X-virulent’ vs. mixed, $p = 0.002$; ‘17X-mild’ vs. mixed, $p < 0.001$). In flexible parametric survival models, with mixed infection as the reference, the ‘17X-virulent’ strain showed a hazard ratio (HR) of 7.5 (95% CI: 1.5 to 38.0; $p = 0.015$); the ‘17X-mild’ strain had an HR of 0.049 (95% CI: 0.011 to 0.21; $p < 0.001$). These imply that relative to mixed strain infections, mild strain infections have ~7.5 times lower risk, and virulent strain infections have ~20 times greater risk, of mortality in *P. yoelii* infections. The incidence rate ratios are also consistent with these other analyses, and suggest approximately 2-fold faster (vs. virulent) and 2-fold slower (vs. mild strain) mortality rates in mixed-strain infections.

3.4. *P. chabaudi* infections

The analysis of mortality in *P. chabaudi* data Dataset III is shown in Table 8. Overall, mixed-strain infections showed a significant difference in risk of mortality relative to single infections: any mixed infections vs. all single (RR = 1.6; 95% CI: 1.1–2.3, $p = 0.009$). While mortality was analyzed within a unified model for all infections, results are presented with differing reference categories within each triad (two single infections and the corresponding mixed infection).

Table 5

Comparison of incubation period in human challenge experiments with *Plasmodium vivax* (Dataset I; N = 184) (RMST, $t^* = 18$ days).

Parasite strain	Incidence rate ratio, (95% CI)	p-Value, IRR	RMST difference (SE)	p-Value, RMST
St. Elizabeth vs. Chesson (ref)	0.80 (0.77–0.83)	<0.001	3.1 (0.25)	0.001
Chesson vs. mixed (ref)	1.1 (1.03–1.15)	<0.001	–1.1 (0.34)	0.001
St. Elizabeth vs. mixed (ref)	0.87 (0.82–0.91)	<0.001	2.0 (0.37)	<0.001

Note: entries in bold are significant with $p < 0.05$.

Table 6
Comparison of time-to-first relapse (from parasite inoculation) in human challenge experiments with *Plasmodium vivax* (Dataset I; N = 66); (RMST, $t^* = 45$ weeks).

Parasite strain	Incidence rate ratio, (95% CI)	p-Value, IRR	RMST difference (SE)	p-Value, RMST
St. Elizabeth vs. Chesson (ref)	0.23 (0.13–0.41)	<0.001	28.1 (2.8)	<0.001
Chesson vs. mixed (ref)	1.9 (0.92–3.9)	0.081	–8.8 (4.4)	0.042
St. Elizabeth vs. mixed (ref)	0.44 (0.27–0.70)	0.001	19.2 (4.3)	<0.001

Note: entries in bold are significant with $p < 0.05$.

Shown first within each triad is the 'control' scenario – that is, a direct comparison between the two single strain infections, followed by comparisons of each single-strain infection to the mixed-strain scenario. In these data, only a single triad shows significant differences between the two 'pure' infections; a significant difference exists between single infections with AS (avirulent) and CB (virulent) strains; and the mixed infection remains significantly different from single-strain AS infections (RR = 0.20; 95% CI 0.58–0.70, $p = 0.012$). However, no evidence for differences was found in comparison with single CB infections (RR = 0.8; 0.59–1.1, $p = 0.162$).

Within the second set of *P. chabaudi* infections (Dataset IV), visual comparison of the Kaplan–Meier curves suggests complex relationships between strains (Fig. 4), as shown by the crossing of the survival curves in many of the infection triads; restricted mean survival times were used for comparisons.

Overall in this set of experiments, mixed infections showed no significant difference in time-to-mortality from single infections: any mixed infection vs. all single-strain infections (Peto–Peto test, $p = 0.214$; RMST difference = -0.5 (SE 0.5), $p = 0.356$); or in risk of mortality relative to single infections (RR = 1.4; 95% CI: 0.86–2.4, $p = 0.168$).

Differences between strains of *P. chabaudi* are shown in Table 9 (dataset IV). As above, we present each infection triad with different reference groups. Several broad trends are apparent in these results. Specifically, in multivariate binary outcome models, mixed infections are indistinguishable from single infections with a more virulent strain, and are significantly different from single infections with less virulent strains.

For example, in triad i., a significant difference exists between single infections with AS (avirulent) and AJ (virulent) strains; and the mixed infection remains significantly different from AS infections (RR = 0.0033; 95% CI 0.000092–0.012, $p = <0.001$). However, no evidence for a significant difference was found when compared with single-strain AJ infections (RR = 2.3; 95% CI: 0.084–6.5, $p = 0.105$). While the RMST differences are consistent with these risk ratios, the Peto–Peto log-rank tests show similar trends but this does not reach significance ($p = 0.068$). In summary, we find that mixed-strain infections are indistinguishable from the single-strain infections with the more

virulent strain, but are significantly different from single-strain infections with the less virulent strain, in cases where there are significant differences between the two single-strain infections. It should be noted that the extremely small reported RRs are due to zero recorded events in some strain categories. The sole outlier to these trends occurs in triad vii., which compares two avirulent strains: AS and CW.

Finally, to complement these two separate analyses of *P. chabaudi* infections, a further comparison was made. All *P. chabaudi* infections from both studies (Datasets III/IV) were compared (N = 160) to assess the overall impact of mixed-strain parasitemia – a limited increase in the risk of mortality from any mixed-strain infections relative to any having any single-strain infection was observed (RR = 1.44; 95% CI 1.04–2.01, $p = 0.026$); as such, infection with any mixed parasite population led to a 44% greater risk of mortality relative to any single-strain infection across a range of parasite clones with varying virulence.

4. Discussion

4.1. Analytical issues

The results of this analysis provide support for the suggestion that the relative contribution of each potential component of observed virulence varies substantially during infections in a strain-specific manner (Metcalf et al., 2012). Specifically, the crossing survival curves and extensive proportional hazard violations imply that the relationships between the time course and virulence measures varies dramatically amongst strains, and these effects are evident even when utilizing different metrics for virulence. Moreover, these findings strongly suggest that measurement of virulence by any single analysis has the potential for biases. Dataset II provides a clear example: there is identical mortality (100%) in the mixed and '7-X virulent' groups, but statistically and biologically significant differences are evident with time-to-event analysis. Additionally, while the effects are generally consistent across the analytical approaches, differences do exist across a single triad. For example, in triad CW/AT (Table 9) shows significant differences when examining the risk of mortality, but no evidence for differences in the analysis of time-to-event (RMST) or survival curves (via Peto–Peto tests).

A second important finding is quantifying the magnitude of strain differences. The largest effect sizes between strains in this study are very biologically significant: differences between single strain infections and infections with mixed-strains using diverse effect measures are modest-to-large for *P. vivax* (1.25 times change in risk for incubation; and 2.3 times risk for relapse), while the maximum for *P. yoelii* in Dataset II are much larger (7.5 and 20.1 times the risk). The range amongst infections with *P. chabaudi* in Dataset III is more limited ranging from 1.7 to 5 times the risk; while the maximum in Dataset IV is ~600 times change in risk but this limit should be interpreted with caution due to the small number of outcome events. These effects range from 'small' to 'very large' by semi-qualitative standards for biological relevance; for example one proposed scale: ~1.5, small; ~2.5, medium; ~4, large; and ~10, very large (Maher et al., 2013). The magnitude of these effects reinforce the potential impact from greater evolutionary thinking in malaria control, drug policy, and 'containment' of resistance (Havryliuk and Ferreira, 2009; Read et al., 2011).

A third analytical aspect is a detailed consideration of the shape of the survival curves in this analysis. In the ecological and demographic

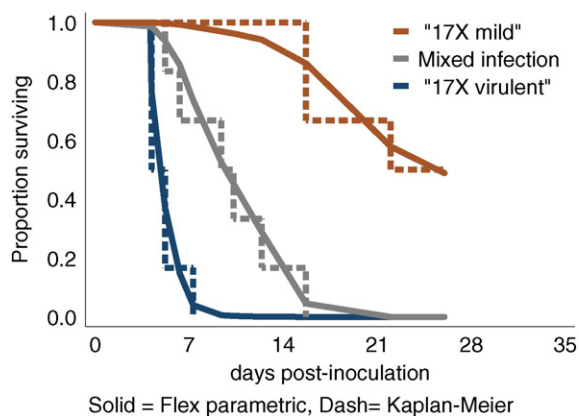


Fig. 3. Kaplan–Meier and flexible-parametric survival model curves comparing time-to-mortality in single strains and mixed-strain infections in murine challenge experiments with *Plasmodium yoelii* (Dataset II; N = 18).

Table 7

Comparison of time-to-mortality in murine challenge experiments with *Plasmodium yoelii* (RMST, $t^* = 22$ days) (Dataset II; N = 18).

Parasite strain	p-value, Peto–Peto test	RMST difference (SE)	p-value, RMST	IRR (95% CI)	p-value, IRR	Hazard ratio (95% CI)	p-value, hazard ratio
'17X-mild' vs. '17X-virulent' (ref)	0.0010	14.9 (1.3)	<0.001	0.51 (0.36–0.72)	<0.001	0.0065 (0.00091–0.046)	<0.001
'17X-virulent' vs. mixed (ref)	0.0103	–4.8 (1.6)	0.002	1.96 (1.39–2.76)	<0.001	7.51 (1.49–37.87)	0.015
'17X-mild' vs. mixed (ref)	0.0034	10.0 (1.9)	<0.001	0.23 (0.075–0.14)	0.004	0.049 (0.011–0.21)	<0.001

Note: entries in bold are significant with $p < 0.05$. IRR = incidence rate ratio.

literature, plots of survival within populations by age, called 'survivorship curves,' allow populations to be categorized into three idealized life courses (referred to as type I, II and III curves) (Pianka, 2011); see Fig. 5. Type III curves are categorized by a large number of mortality events early in the time course; type II by a relatively constant rate of events; and type I by generally low 'failure' rate until the oldest age classes.

Examination of the survival curves within this study (especially *P. yoelii*) and Fig. 1 (with allowances for the crossing curves) closely mimics these classes of survivorship curves, although the underlying mechanisms are unrelated. We suggest that future studies should consider this general scheme for classification of virulence for two reasons. First, it allows examination for study population-level effects and 'smooths-out' individual-level variation in response. Secondly, it summarizes the complex interplay between host and parasites to more comprehensively capture the composite endpoint of 'virulence.' Finally, several different metrics have been suggested to allow quantitative comparisons between the convexity of survivorship curves, and have potentially important utility in directly classifying and comparing the virulence of parasite populations (Anson, 2002).

4.2. Concordance between studies and potential drivers of virulence

Early studies included a small set of parasite co-inoculations in neurosyphilis patients (Boyd et al., 1941, 1938) which suggested that co-inoculation led delayed parasite clearance, and impaired acquisition of immunity to either strain as assessed by subsequent re-infections; however, no direct measures of virulence were reported.

The authors of the original study with mixed *P. vivax* inoculations (dataset I) in human populations analyzed in this work concluded that within wide individual variation, the overall pattern of all recorded relapses in mixed-strain infections was a combination of the two strain-

specific responses; however, these conclusions were based solely on a qualitative assessment of the overall respective clinical courses; incubation period was not considered (Cooper et al., 1950).

We find that the incubation period shows significant differences between the mixed and each single-strain infection. Consideration of the time-to-first relapse, however, suggests that the mixed strain infections are only marginally different (RMST analysis) or indistinguishable (IRR analysis) from single-strain infections with the virulent Chesson strain. Although poorly understood, hypnozoite re-activation is a different biological process from incubation (Markus, 2012), so the results from the relapse analysis may not be directly comparable to other measures of virulence.

Qualitative comparison of the survival curves in Fig. 2 suggests that the earlier portion of the mixed-infection curve more closely follows, but is not equivalent to, that of Chesson strain infections. However, we are unable to differentiate between two potential mechanisms: coordinated relapse between genetically diverse hypnozoites, or simply the first result of having two superimposed hypnozoite relapse curves.

Consensus about clonal diversity within relapses has been elusive (Imwong et al., 2012, 2007), and conclusions may be highly dependent on the specific probes used to compare parasite-relatedness (Restrepo et al., 2011); however, it is clear that some parasite sub-populations that are more prone to relapse, and that there is extensive diversity in parasite populations (Battle et al., 2014; Lin et al., 2012; Lover and Coker, 2013). Critically, relapse epidemiology has been highlighted as a key gap in control of this parasite (Ferreira et al., 2007; Havryliuk and Ferreira, 2009).

In discussion of the mixed murine *P. yoelii* infections (Dataset II) the original study authors note that while the mild strain preferentially infected only reticulocytes even in fatal outcomes, and the virulent form 'overwhelmingly' infected mature erythrocytes, mixed infections initially showed parasitemias in both RBC stages, which then progressed in the four surviving animals to be predominately composed of infected reticulocytes (Hargreaves et al., 1975). Our results quantify the size of these differences, and finding an intermediate survival curve in mixed-strain infections provides further support for these hematological observations.

Earlier work using *P. chabaudi* found greater virulence in mixed-strain infections relative to single clone infections, as measured by weight loss and RBC count (anemia level) (Taylor et al., 1998). Our analyses of *P. chabaudi* data do not show a significant difference in mortality between mixed infections and all single strains in these data, but this is potentially due to the range of parasite clones examined. Moreover, our results suggest that within these parasite lineages, RBC and weight loss metrics may only capture a portion of the complexity in the measurement of 'virulence'; within parasite triads, we find mortality in mixed-strain infections is not increased, but equivalent to mortality in infections with the more virulent strain in *P. chabaudi* infections. In examination of the strains that appear in both *P. chabaudi* studies (AS/CB; datasets III/IV) it is noteworthy that while the direction of the effects is consistent, the size of the risk ratios differs by ~100-fold. While many experimental differences are evident (different laboratories and mouse strains, decades apart) these differences are remarkably consistent with the inocula (10^4 vs. 10^6 parasitized erythrocytes). The broad consistency of effect sizes between strains in the other experiment in this analysis (including mosquito-transmitted infections) could indicate

Table 8

Comparison of risk ratios from binomial models for mortality in mixed infection by strains in murine challenge experiments with *Plasmodium chabaudi* (Dataset III; N = 60).

Parasite strain triad (more virulent strains in bold)	Comparison	Risk ratio (95% CI)	p-value, risk ratio
i. Mixed AS/ CB (ref)	AS vs. CB (ref)	0.25 (0.069–0.91)	0.035
	AS vs. AS + CB (ref)	0.20 (0.58–0.70)	0.012
	CB vs. AS + CB (ref)	0.80 (0.59–1.1)	0.162
	DS vs. CB (ref)	0.75 (0.41–1.4)	0.346
ii. Mixed DS/ CB (ref)	DS vs. DS + CB (ref)	0.60 (0.36–0.99)	0.050
	CB vs. DS + CB (ref)	0.80 (0.59–1.1)	0.162
	AS vs. DS (ref)	0.33 (0.09–1.3)	0.111
	AS vs. AS + DS (ref)	0.33 (0.09–1.3)	0.111
iii. Mixed AS/ DS (ref)	DS vs. AS + DS (ref)	1.0 (0.49–2.1)	1.0

Note: entries in bold are significant with $p < 0.05$.

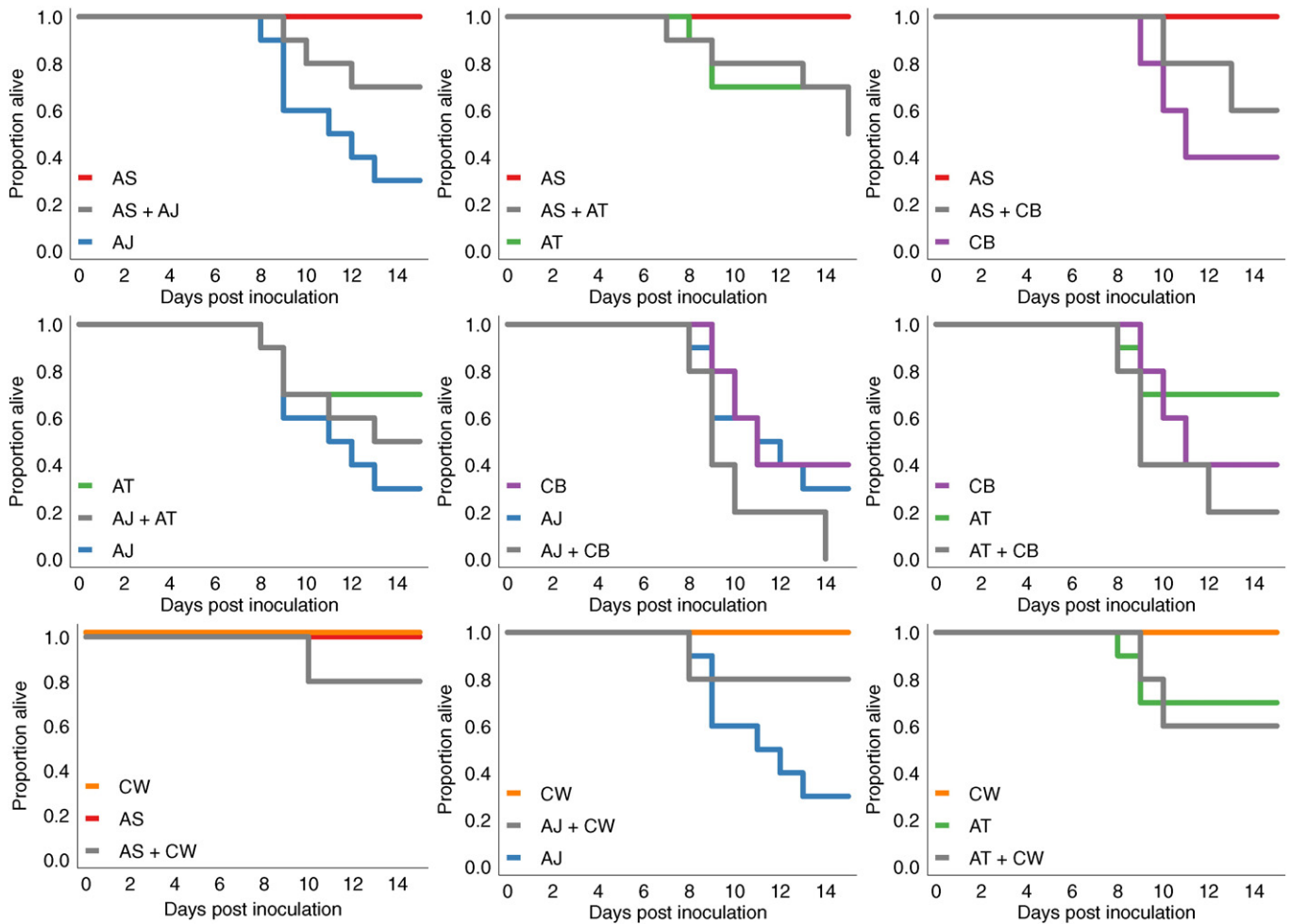


Fig. 4. Kaplan–Meier curves comparing time-to-mortality in single strains and mixed-strain infections in murine challenge experiments with *Plasmodium chabaudi* (Dataset IV; N = 100).

that exposure to 10^6 parasitized erythrocytes might produce extreme parasite dynamics.

Our results reconcile these *P. chabaudi* experiments with mathematical models that have suggested that host mortality is strongly driven by the more virulent strain within an infection (Alizon and van Baalen, 2008), but suggest the existence of a fundamental limit. Comparison of the risk ratios between the CB/AS triad (RR = 2.5) and all mixed/pure infections (RR = 1.44) amongst all studied strains, supports the findings of Alizon which suggest that the antigenic distance between strains has critical impacts on observed virulence (Alizon and van Baalen, 2008). These mathematical models suggest that there may be a ‘sweet-spot’ where maximum competition occurs – if strains are highly antigenically divergent, they can parasitize essentially independent niches in the host with subsequent limited need for competition; whereas highly related parasites are too similar to be differentiated by general immune responses. The overall comparisons of single-strains as survival curves or as RR in these studies provide generally consistent results with those from comprehensive models of propagation number in studies with many of the same *P. chabaudi* strains (Metcalf et al., 2012).

In our analysis of the much larger set of experiments using *P. chabaudi* we find greater nuances in interactions between strains, and we find that mortality and time-to-event in mixed-strain infections are both *indistinguishable* from those of virulent-strain single infections. This is in sharp contrast to the results from *P. yoelii* experiments, with similar triad-wise sample sizes. One potential explanation for these

differences comes from differential blood cell preferences: *P. vivax* and *P. yoelii* generally infect the youngest RBC stages (reticulocytes) (McKenzie et al., 2002), whereas *P. chabaudi* (like *P. falciparum*) has limited specificity, and readily infects both reticulocytes and normocytes (Snounou et al., 1992). These results concur with those from modeling studies that explicitly modeled the impact of age-structured erythrocyte susceptibility; the authors concluded that while this clearly is an important modulator of parasite dynamics, more research into these effects should be a priority (McQueen and McKenzie, 2004).

These divergent dynamics reinforce the suggestion that virulence has different underlying mechanisms in *P. vivax*-like and *P. falciparum*-like parasites (Wassmer et al., 2015). This hypothesis is consistent with data suggesting that immunity and RBC dynamics, which differ greatly between the parasite ‘types,’ are both important contributors to competitive ability and parasite dynamics. This suggests that maximal competitive advantage may have a fundamental limit, driven primarily by limited numbers of susceptible RBCs in *P. vivax*-type infections, so virulent strains can only out-compete to a certain extent, but other interrelated factors may also play an important role (Alizon et al., 2009). However, other studies with *P. chabaudi* reached differing conclusions and suggest the evolutionary trajectory of higher-virulence strains within a mixed-strain infection are primarily impacted by burst size (number of progeny per merozoite) plus differential interactions with host immunity (Mideo et al., 2011). The complexity of the interplay between these factors was observed in observational studies of avian infections, where wild-caught specimens with mixed-strain

Table 9

Comparison of Kaplan–Meier estimator, restricted mean survival times, and risk ratios from binomial models for mortality in mixed infection by strains in murine challenge experiments with *Plasmodium chabaudi* (Dataset IV; N = 100); (RMST, t* = 15 days).

Parasite strain triad (more virulent strains in bold)	Comparison	p-value, Peto–Peto test	RMST difference (SE)	p-Value RMST difference	Risk ratio (95% CI)	p-value, risk ratio
i. Mixed AS/AJ	AS vs. AJ (ref)	0.0015	3.4 (0.84)	<0.001	0.0015 (0.00054–0.0038)	<0.001
	AS vs. AS + AJ (ref)	0.0679	1.4 (0.71)	0.049	0.0033 (0.00092–0.012)	<0.001
	AJ vs. AS + AJ (ref)	0.0792	–2.0 (1.1)	0.070	2.32 (0.84–6.5)	0.105
ii. Mixed AS/AT	AS vs. AT (ref)	0.0685	1.9 (0.93)	0.041	0.0033 (0.00091–0.012)	<0.001
	AS vs. AS + AT (ref)	0.0130	1.6 (0.89)	0.072	0.0020 (0.00068–0.0059)	<0.001
	AT vs. AS + AT (ref)	0.5584	–0.30 (1.3)	0.816	0.61 (0.19–1.88)	0.381
iii. Mixed AS/CB	AS vs. CB (ref)	0.0062	3.0 (1.2)	0.012	0.0018 (0.00054–0.0056)	<0.001
	AS vs. AS + CB (ref)	0.0350	1.4 (0.94)	0.145	0.0026 (0.00063–0.011)	<0.001
	CB vs. AS + CB (ref)	0.3547	–1.6 (1.4)	0.266	1.50 (0.41–5.5)	0.539
iv. Mixed AJ/AT	AJ vs. AT (ref)	0.1933	–1.5 (1.3)	0.232	2.32 (0.83–6.55)	0.109
	AJ vs. AJ + AT (ref)	0.4769	–0.90 (1.2)	0.463	1.40 (0.67–2.9)	0.371
	AT vs. AJ + AT (ref)	0.5080	0.60 (1.3)	0.641	0.61 (0.19–1.9)	0.379
v. Mixed AJ/CB	AJ vs. CB (ref)	na	–0.43 (1.4)	0.762	1.2 (0.52–2.86)	0.652
	AJ vs. AJ + CB (ref)	0.2534	1.6 (1.3)	0.222	0.74 (0.45–1.2)	0.184
	CB vs. AJ + CB (ref)	0.1293	2.0 (1.5)	0.176	0.60 (0.30–1.2)	0.163
vi. Mixed AT/CB	AT vs. CB (ref)	na	1.1 (1.5)	0.470	0.52 (0.16–1.7)	0.290
	AT vs. AT + CB (ref)	0.1245	2.5 (1.5)	0.099	0.39 (0.14–1.1)	0.084
	CB vs. AT + CB (ref)	0.3602	1.4 (1.6)	0.388	0.75 (0.32–1.7)	0.503
vii. Mixed AS/CW	AS vs. CW (ref)	0.1573	0.33 (0.32)	0.917	0.06 (0.20–4.5)	0.955
	AS vs. AS + CW (ref)	0.1573	1.0 (0.95)	0.279	0.0048 (0.00066–0.035)	<0.001
	CW vs. AS + CW (ref)	0.3173	1.0 (0.90)	0.266	0.0050 (0.00058–0.043)	<0.001
viii. Mixed CW/AJ	CW vs. AJ (ref)	0.0230	3.4 (0.93)	<0.001	0.0015 (0.00039–0.0057)	<0.001
	CW vs. CW + AJ (ref)	0.3173	1.4 (1.3)	0.266	0.0050 (0.00058–0.043)	<0.001
	AJ vs. CW + AJ (ref)	na	–2.0 (1.6)	0.209	3.3 (0.54–20.7)	0.196
ix. Mixed CW/AT	CW vs. AT (ref)	0.1952	1.9 (1.1)	0.067	0.0035 (0.00070–0.017)	<0.001
	CW vs. CW + AT (ref)	0.1360	2.2 (1.2)	0.071	0.0050 (0.00058–0.043)	<0.001
	AT vs. CW + AT (ref)	na	0.33 (1.6)	0.084	1.4 (0.19–10.9)	0.729

Notes: (RMST, at t* = 15 days); entries in bold are significant with p < 0.05; 'na' signifies that test is not applicable due to crossing survival curves.

parasitemia had larger numbers of ectoparasites and decreased reproductive success (Marzal et al., 2008).

In spite of analytical limitations in these data, our results suggest that general virulence proxies or single methodologies may fail to capture all facets of observed virulence, and a broader selection of endpoints and methods should be considered in analyses.

4.3. Limitations

The *P. vivax* and *P. yoelii* studies are based on analysis of secondary data with limited experimental detail reported. Additionally, the murine experiments used blood-transfer via syringe and not infected vectors, which has the potential to impact parasite dynamics (Nkhoma et al., 2012; Paul et al., 2004) and studies have suggested that parasite passage through anopheline vectors can modulate virulence (Spence et al., 2013). Therefore syringe-induced infections likely represent extremes in virulence that may not reflect natural dynamics (Mideo et al., 2011). Lastly, many of the comparisons in the murine models were made using a small number of animals per treatment

group, and not finding an effect in some groups may be due to sample size limitations.

5. Conclusions

These results link human and murine experimental studies, and also provide further evidence for differing dynamics between malaria species dependent on blood-stage cell specificities. These 'slippages' suggest research areas that could provide important insights into intra-host malaria dynamics, and should be prioritized in planning new murine challenge studies. Our results suggest at least some of these conflicting results could be partially addressed by consideration of a more diverse set of endpoints beyond comparisons of mean anemia or parasitemia between groups and the use of effect size measures as opposed to p-values of significance. While constrained by crossing curves and zero-event categories, our analyses suggest a potential for fuller use of data that could maximize the utility of these complex and experimentally demanding experiments.

While virulence management has been highlighted as a potential key contribution from evolutionary biology especially in malaria, this paradigm has had limited impact on clinical or epidemiological practice (Restif, 2009). The effect sizes measured between single strain and mixed-strain inoculations in these studies are highly biologically significant with magnitudes that are well within the 'important' range for epidemiological studies; these findings reinforce the importance of greater consideration of evolutionary epidemiology in malaria control.

While the inherent limitations of these model systems in other host species have been recognized, they provide invaluable insight into parasite evolutionary dynamics within a host, and a recent comprehensive review concluded that there needs to be closer linkages between human studies and animal models to allow the development of truly synergistic research programs (Langhorne et al., 2011).

Finally, these results reinforce that evolutionary drivers of strain competition and virulence differ in fundamental ways between *P. vivax* and *P. falciparum*. This suggests several broad avenues for future

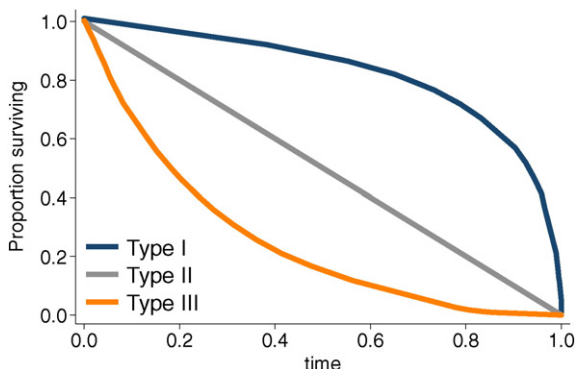


Fig. 5. Idealized ecological/demographic survivorship curves.

animal models. A head-to-head comparison within the same laboratory between reticulocyte-specific and generalist parasites encompassing similar differences in virulence could provide important insights into virulence and parasite evolution. These results could be of critical importance for the planning of species-specific policies for drug-resistance mitigation, and towards global malaria elimination for all malaria species.

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Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AAL envisioned the study, collected and analyzed all data, and wrote the first draft; RJC supervised throughout and edited the drafts.

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