# The effects of host immunity on virulence–transmissibility relationships in the rodent malaria parasite *Plasmodium chabaudi*

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SUMMARY

Here we examined the impact of host immunity on relationships between parasite virulence, transmission rate, intrinsic growth rate and host recovery rate in the rodent malaria parasite, *Plasmodium chabaudi*. Groups of naïve and immunized mice were infected with 1 of 10 cloned lines of parasites and their infection dynamics were monitored for 19 days. We found that (1) host immunity reduced the growth rate, virulence, transmission rate and infection length, with a consequent 3-fold reduction in life-time transmission potential, (2) clone means for these traits ranked similarly across naïve and immunized mice, (3) regression slopes of transmission potential on growth rate, virulence and infection length were similar in naïve and immunized mice, (4) virulence and infection length were positively correlated in immunized but not naïve mice, and (5) for a similar level of parasite growth rate and virulence, transmission potential and infection length were lower in immunized than naïve mice. Thus host immunity reduced all these fitness traits in a manner consistent with direct parasite-driven biological links among them. These results support the basic assumption underlying our theory that predicts that anti-disease vaccines will select for higher virulence in those microparasites for which virulence is integrally linked to transmission.

Key words: malaria, virulence, transmission, evolution, immunity, recovery rate.

## INTRODUCTION

Host immunity provides protection against parasites. Two forms of protection for individual hosts may be distinguished: one against the establishment of an infection (anti-infection), the other against damage to the host once the infection is established (anti-disease). In vertebrates, acquired immunity typically provides anti-disease resistance, often by reducing the rate of in-host replication of the parasite. Immunity can also reduce parasite transmission, thus alleviating transmission pressure and hence disease burden in the general population (so-called herd immunity, Anderson & May, 1991). The success of vaccination in combating many of the world's infectious diseases testifies to this dual effect of immunity (Plotkin & Orenstein, 1999). However, if, in the absence of host death, more virulent strains have greater fitness (transmission) than less virulent strains, evolutionary theory predicts that parasites will evolve higher intrinsic virulence in response to anti-disease vaccination programmes (Gandon *et al.* 2001). This is because semi-immunity both reduces transmission and reduces selection against virulence (virulent strains are less likely to kill immunized hosts and therefore themselves). Thus natural selection will favour increased intrinsic virulence, this up to the point when the fitness cost of virulence through premature death outweighs the fitness benefit through transmission. This prediction is general for any form of host defence that operates on the virulence–transmissibility relationship.

A critical assumption of this model is that there is a genetically determined, positive and saturating relationship between transmission and virulence which is maintained across different levels of host defence. While there are some data supporting the first part of this assumption for microparasites (e.g. Fenner & Ratcliffe, 1965; Anderson & May, 1982; Diffley *et al.* 1987; Bull, Molineux & Rice, 1991; Turner, Aslam & Dye, 1995; Lipsitch & Moxon, 1997; Mackinnon & Read, 1999*a*, *b*; Eisen & Schall, 2000), data on how transmission and virulence are related in less- versus well-defended hosts are rare (Best & Kerr, 2000). In the present study we examined this assumption in the rodent malaria parasite, *P. chabaudi*, in laboratory mice. We examined 10 parasite clones for their in-host growth rate, virulence, transmission potential and infection length when infecting naïve and immunized mice. Our aims were to determine whether (1) host immunity reduced all 4 parasite fitness traits, (2) clones ranked consistently in naïve and immunized hosts, and (3) relationships among traits differed between naïve and immunized hosts.

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## MATERIALS AND METHODS

### *Experimental procedures*

We used the rodent malaria, *P. chabaudi*, as a laboratory model because of its similarity in infection characteristics to the most virulent of the human malarias, *P. falciparum* (e.g. rapid asexual growth rate followed by a peak of transmission, partial sequestration, incomplete immunity that is partly strain-specific, and clonal antigenic variation (Cox, 1988)). The natural host of *P. chabaudi* is the thicket rat, *Thamnomys rutilans*, a sibling genus of the laboratory mice used in these studies (Ellerman, 1940). In this experiment, inbred C57Bl/6J male mice were used, aged 5–7 weeks at the time of first infection. Mice were housed in standard conditions at 21 °C with food provided *ad libitum* (diet 41B, Harlan, UK) and 0·05% para-aminobenzoic acid added to their drinking water. Light was provided between 8.00 h and 20.00 h (GMT).

Using standard infection procedures (Taylor, Walliker & Read, 1997*a*), groups of 5 naïve and 5 immunized mice were infected by intraperitoneal injection of $10^6$ asexual parasites per mouse from 1 of 10 cloned parasite lines in 2 separate experimental blocks (5 clones per block, see below). Mice had been immunized by inoculation 3 weeks previously with $10^4$ parasites of clone ER followed by oral drug treatment with mefloquine (12·5 mg/kg) for 3 days from day 4 post-infection (p.i.) (i.e. 1–2 days before the infection becomes patent). Previous experiments had established that this infection-cure regime would generate the partial immunity required for these experiments, as compared with the near-sterilizing immunity that would occur had mice of this genotype been left to self-cure. No mice demonstrated detectable parasitaemia prior to re-challenge. Naïve mice were not inoculated but were drug treated at the same time. Clones were originally derived by serial dilution of isolates obtained from wild-caught hosts and were all assigned to the *P. c. chabaudi* subspecies (Beale, Carter & Walliker, 1978). They are denoted AD, AL, AT, CW, ER (block 1), AJ, AQ, AS, BC and CQ (block 2). All clones had undergone fewer than 10 serial passages in mice prior to use in these experiments, with the exception of ER for which the passage history is unknown.

Every 1–2 days until day 19 p.i., the parasitaemia and gametocytaemia (proportion of red blood cells infected with asexual and sexual forms of parasites, respectively), and the red blood cell (RBC) density and liveweight of the mouse were measured using routine procedures (Taylor, Mackinnon & Read, 1998). Asexual parasites are the numerically dominant haploid forms of the parasite that replicate in RBCs approximately 8-fold every 24 h in a synchronous fashion and cause pathology, primarily anaemia. Gametocytes are the non-replicating haploid sexual forms that transmit to mosquitoes and are derived by differentiation of asexual forms at a rate of around 0·1–10% per replication cycle (Buckling, Crooks & Read, 1999).

### *Statistical analyses*

Four groups of 3 traits each were derived from the infection data, classified as Growth, Virulence, Transmission and Recovery rate traits: these are described in Table 1. In addition to the 12 individual traits, the first principal component of each group of traits was calculated in order to combine related traits into a single representative variable (see below). Individual traits were chosen to reflect either key events in the infection (e.g. maxima or minima) or to summarize the entire infection (e.g. average over time). To avoid generating artificial relationships among traits through sharing the same numerator or denominator (e.g. RBC density) and hence measurement errors, we chose traits that were calculated independently of each other. Measurement of life-time transmission to mosquitoes was not feasible: instead we measured life-time gametocyte production as an indicator of transmission potential since gametocyte density correlates to the probability of transmission to mosquitoes (reviewed by Taylor & Read, 1997).

The statistical analyses were performed using SAS (SAS, 1990). All analyses were performed on individual traits as well as on the first principal components of groups of traits: the latter were obtained using the PROC PRINCOMP procedure on combined data from naïve and immunized mice. Where necessary, transformations were performed to normalize the data prior to analysis (Table 1).

To address the question of whether immunity reduced growth, virulence, transmission potential and recovery rate, an analysis of variance (PROC MIXED) was performed fitting fixed effects for experimental block and treatment (immunized versus naïve), an interaction between these where significant ($P < 0·05$), and a random effect for clone. Significance tests for fixed effects were performed using F-ratios.

To determine whether there were significant differences between clones within treatments, variance component analyses were used to partition the total variance into its between-clone and within-clone components. This was done using the PROC MIXED procedure fitting a model with experimental block as a fixed effect and clone as a random effect. Repeatability was calculated as the ratio of the between-clone variance to the total variance after adjustment for block effects. These analyses were performed within treatments because variances differed considerably between treatments. Significance tests for clone variation were determined by comparing twice the difference in log likelihood values from models with and without clone included to a chi-squared distribution with 1 degree of freedom.

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Table 1. Description of traits relating to asexual population growth rate, virulence, transmission and recovery rate of mice infected with *Plasmodium chabaudi*

(Maximum RBC loss and weight loss are the initial values on day 0 p.i. minus the minimum reached during the infection. Rate of weight loss is the maximum weight loss divided by the number of days to reach the minimum. Maximum gametocyte conversion ratio (the ratio of asexual parasites that give rise to gametocytes) was calculated from the formula given in Buckling *et al*. (1999) which assumes a 2-day gametocyte maturation time. Length of infection was defined as the number of days until the infection reached undetectable levels, i.e. zero parasites were found in a count of around $10^5$ cells. Estimates of this rate of decline were obtained by fitting a model with a random effect for the intercept and slope for each mouse through time (days post-peak) to data on the natural logarithm of parasite density, allowing for correlated errors on the same mouse. If the mouse had not reached zero parasitaemia by the last day of measurement (day 19), its data were excluded from analyses involving this trait. The first principal component explained 79–92% of the variation among traits of the same type with the average correlation among traits of the same type (across clones and treatments) being 0·75 (range of 0·52 to 0·99).)

| Trait | Trait type | Trait code | Transformation of trait ($x$) |
|---|---|---|---|
| Parasitaemia on day 5 p.i. | Growth | G1 | Arcsine $\sqrt{x}$ |
| Maximum parasitaemia | Growth | G2 | Arcsine $\sqrt{x}$ |
| Average parasitaemia while patent | Growth | G3 | $\mathrm{Log}_{10}(x+0{\cdot}001)$ |
| Maximum RBC loss | Virulence | V1 | None |
| Maximum weight loss | Virulence | V2 | None |
| Rate of weight loss | Virulence | V3 | None |
| Maximum gametocytaemia | Transmission | T1 | Arcsine $\sqrt{(100x+0{\cdot}001)}$ |
| Average gametocytaemia while patent | Transmission | T2 | $\mathrm{Log}_{10}(100x+0{\cdot}001)$ |
| Maximum conversion ratio | Transmission | T3 | $\mathrm{Log}_{10}(100x+0{\cdot}1)$ |
| Total length of infection | Recovery rate | R1 | None |
| Number of days patent after peak | Recovery rate | R2 | None |
| Rate of decline in parasite density | Recovery rate | R3 | Linear regression of $\ln(x+0{\cdot}01)$ on day post-peak |
| First principal component for growth rate | Growth | PC-G | None |
| First principal component for virulence | Virulence | PC-V | None |
| First principal component for transmission | Transmission | PC-T | None |
| First principal component for recovery rate | Recovery rate | PC-R | None |

To determine whether a clone performed in a consistent way across treatments, Pearson correlations and Spearman Rank correlations between estimates of clone means obtained from the within-treatment analyses described above were calculated. A further test for clone by treatment interactions (i.e. whether clones performed inconsistently across treatments) was performed by fitting a model to data from both treatments with fixed effects for block and treatment, and random effects for clone and clone by treatment. As above, likelihood ratio tests were performed for the statistical significance of clone by treatment effects with and without this term in the model.

To determine whether immunity altered the relationships among growth, virulence, transmission and recovery rate traits, linear regression analyses were performed using PROC GLM. For pairs of traits where one was an independent variable and the other a regressor variable, a model was fitted with fixed effect factors for block and treatment, a continuous linear covariate for the regressor, and an interaction between treatment and regressor. This model thus allowed testing of differences between treatments in the slopes of the linear regression line (significance of the interaction term), and a difference between the heights of the intercepts (significance of the treatment term). The difference between the height of the regression lines at the mean value of the regressor variable (over both treatments) was also tested for statistical significance using the 'ESTIMATE' option. The reason for doing this was to determine whether the separate regression lines for the 2 treatments 'joined up' at their point of maximum overlap for the regressor trait: if so, this would indicate that the 2 lines could form part of the 1 curve, and hence that there may be a single unifying relationship between the traits across both treatments. A 'curvilinear' model was also fitted which included a quadratic term for the regressor within each treatment. As in the linear model, a test was made for whether the heights of the regression lines differed at the mean value of the regressor variable. The linear model was the default when the quadratic model detected no significant ($P>0{\cdot}05$) quadratic effect in both the treatment groups.

## RESULTS

Immunized mice had significantly lower parasitaemias, gametocytaemias, conversion ratios, RBC and liveweight loss, and recovered more quickly from the infection than naïve mice (Fig. 1, Table 2). The estimated total number of gametocytes produced during the infection averaged $17{\cdot}5 \pm 15{\cdot}1 \times 10^6$/ml in naïve mice compared with $6{\cdot}3 \pm 7{\cdot}6 \times 10^6$/ml in immunized mice which was highly significantly different when analysed on the log transformed scale ($P<0{\cdot}001$). Two naïve mice died on day 9 p.i., 1 infected with clone BC and the other with clone ER. By day 19 p.i., 4 naïve mice (3 infected with ER and 1

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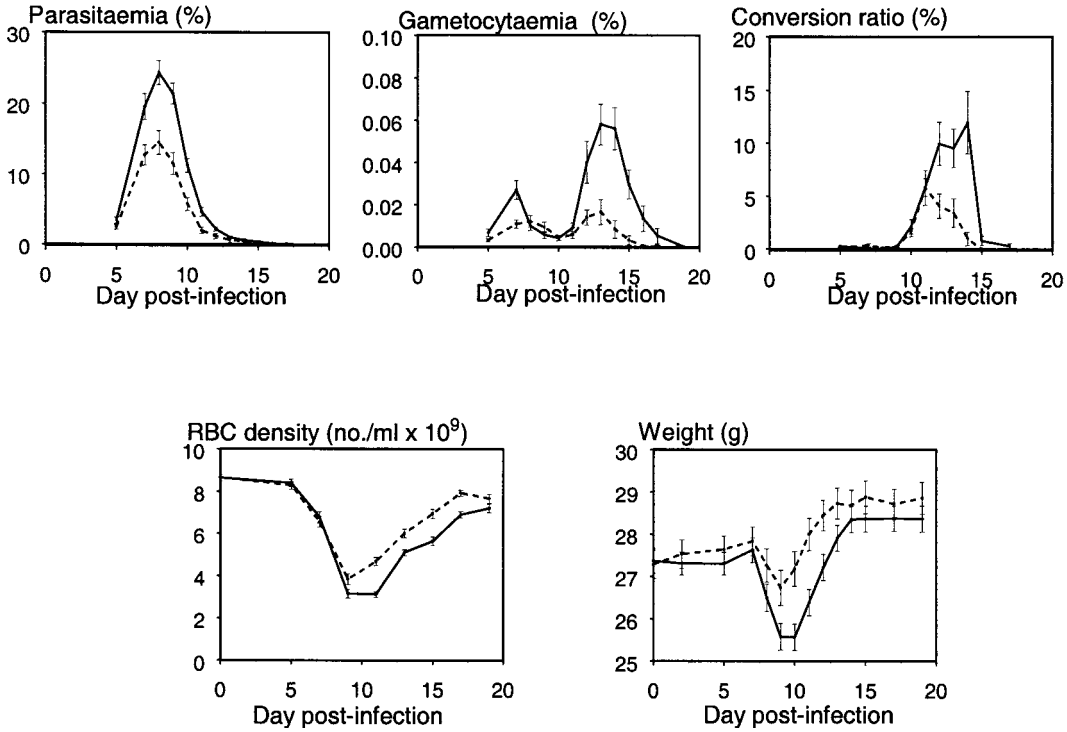


Fig. 1. Infection profiles of naïve (solid lines) or immunized (broken lines) mice infected with *Plasmodium chabaudi*. Each line represents the mean (and S.E.) of 45–49 mice infected with 1 of 10 different parasite clones.

with AJ) and 2 immunized mice (1 infected with ER and 1 with AJ) all had less than 0·5%, but still detectable, parasitaemia.

There was significant clone variation for all traits, i.e. clones were repeatable in their performance across mice infected with the same clone within the same treatment (Table 2). Repeatability estimates were generally lower in immunized mice than in naïve mice: this was due to higher phenotypic, rather than lower genetic (between-clone) variances, and was probably a reflection of extra variability introduced by the stochastic events involved with the immunization procedure. Pearson correlations between a clone's performance in naïve mice and immunized mice (repeatabilities across treatments) were positive and moderate to high (though generally not significant) for all traits except maximum gametocytaemia (Table 2): thus, in general, clones ranked consistently in performance in naïve and immunized mice (Fig. 2). Significant clone by treatment interactions were found for maximum gametocytaemia (T1, $P<0·05$), the first principal component for transmission traits (PC-T, $P<0·05$), rate of weight loss (V3, $P<0·01$) and the first principal component for virulence (PC-V, $P<0·05$). However, these interactions mainly reflected differences between treatments in the magnitude, rather than rank, of clones' performances (Fig. 2). Spearman rank correlations were similar to Pearson correlations (data not shown).

Phenotypic (across-mouse) and genetic (across-clone) regression slopes for growth, virulence and transmission traits were all positive and similar in naïve and immunized mice (Table 3, Fig. 3). Thus the magnitude of the relationship among these traits was not altered by immunity. Regression slopes were almost always less than unity on the standardized scale: thus a unit increase in growth or virulence produced less than a unit of increase in transmission potential. Genetic relationships mirrored phenotypic relationships though generally were less significant (Table 3). Results for pairwise analyses of individual traits (not shown) were accurately reflected by the first principal component of the group of traits (Table 3) indicating that the results did not depend on particular trait definitions. An exception to this was that maximum conversion ratio (T3) did not correlate to virulence traits ($P>0·4$) in naïve or immunized mice whereas other transmission traits did (usually $P<0·01$). Another exception was that average gametocytaemia (T2) did not correlate ($P>0·15$) with recovery rate traits in immunized mice, while other transmission traits did ($P<0·01$). For only 3 of the 54 phenotypic regression estimates on individual traits was there a significant ($P<0·05$) quadratic effect: no quadratic effects were detected in the analyses of first principal components or in the genetic regression analyses of individual traits.

In contrast to the slopes, the heights of the regression lines of transmission potential on growth and virulence, as represented by their principal components, were significantly greater in naïve than in immunized mice by around 0·5–1 standard deviation (Tables 2 and 3, Fig. 3). Thus, for a given growth rate and virulence, transmission potential was lower in

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Table 2. Means, standard deviations and clone effects on the performance of *Plasmodium chabaudi* clones when measured in naïve and immunized mice for traits relating to growth, virulence, transmission and recovery rate (see Table 1)

(Means and standard deviations are given on the raw scale, $n=45–49$ per treatment. Figures under the heading 'Clone effects' are the proportions of variation explained by clone within treatment, and the Pearson correlations between clone means across treatments, i.e. the within-treatment and across-treatment repeatabilities. Significance levels next to the means pertain to differences between naïve and immunized mice when analysed on the transformed scale. Significance levels next to repeatabilities test whether the estimate is different from zero. † $P<0{\cdot}10$, * $P<0{\cdot}05$, ** $P<0{\cdot}01$, *** $P<0{\cdot}001$.)

| | | | | | Clone effects | |
|---|---|---|---|---|---|---|
| Trait code | Treatment | Units | Mean | S.D. | Within treatments | Across treatments |
| G1 | Naïve | % | 3·44 | 3·00 | 0·66*** | |
| | Immunized | | 2·47* | 2·49 | 0·34** | 0·78** |
| G2 | Naïve | % | 30·1 | 11·4 | 0·35** | |
| | Immunized | | 18·2*** | 13·0 | 0·30* | 0·62† |
| G3 | Naïve | %/day | 6·03 | 1·97 | 0·45*** | |
| | Immunized | | 4·21*** | 2·64 | 0·25* | 0·35 |
| V1 | Naïve | $\times 10^9$/ml | 6·12 | 1·06 | 0·46** | |
| | Immunized | | 5·28*** | 1·03 | 0·43* | 0·49 |
| V2 | Naïve | g | 2·43 | 1·58 | 0·33** | |
| | Immunized | | 1·05*** | 2·10 | 0·45*** | 0·43 |
| V3 | Naïve | g/day | 0·26 | 0·17 | 0·35** | |
| | Immunized | | 0·12*** | 0·22 | 0·44** | 0·41 |
| T1 | Naïve | % | 0·039 | 0·037 | 0·68*** | |
| | Immunized | | 0·012*** | 0·020 | 0·34** | 0·05 |
| T2 | Naïve | %/day | 0·018 | 0·015 | 0·78*** | |
| | Immunized | | 0·008*** | 0·008 | 0·30* | 0·65* |
| T3 | Naïve | % | 21·8 | 2·00 | 0·42** | |
| | Immunized | | 11·3*** | 0·91 | 0·12 | 0·56† |
| R1 | Naïve | Days | 16·4 | 1·57 | 0·65*** | |
| | Immunized | | 13·9*** | 2·23 | 0·42** | 0·67* |
| R2 | Naïve | Days | 8·82 | 1·47 | 0·49** | |
| | Immunized | | 6·21*** | 2·24 | 0·36** | 0·74* |
| R3 | Naïve | $\times 10^9$/ml/day | −0·60 | 0·11 | 0·43** | |
| | Immunized | | −0·80*** | 0·31 | 0·25† | 0·23 |
| PC-G | Naïve | None | 0·63 | 1·20 | 0·53*** | |
| | Immunized | | −0·60*** | 1·67 | 0·26* | 0·47 |
| PC-V | Naïve | None | 0·64 | 1·28 | 0·36** | |
| | Immunized | | −0·65*** | 1·62 | 0·49*** | 0·48 |
| PC-T | Naïve | None | 0·76 | 1·32 | 0·69*** | |
| | Immunized | | −0·75*** | 1·35 | 0·25† | 0·45 |
| PC-R | Naïve | None | −0·89 | 0·73 | 0·34* | |
| | Immunized | | 0·68*** | 1·85 | 0·36** | 0·27 |

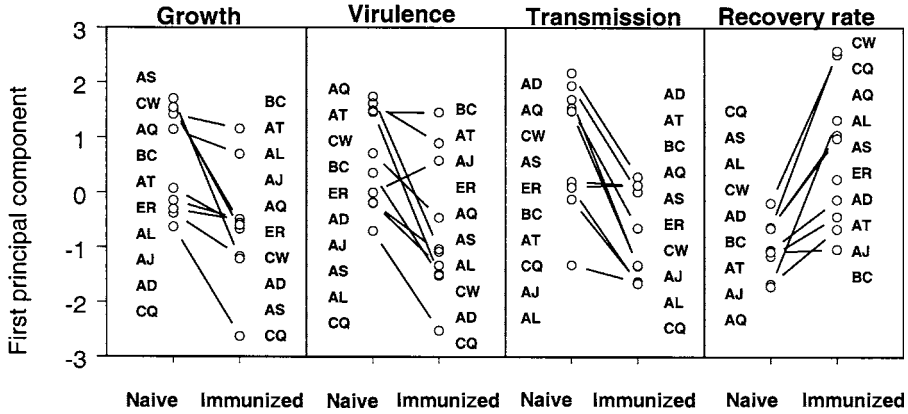


Fig. 2. Performance of clones of *Plasmodium chabaudi* in naïve and immunized mice for traits relating to growth rate, virulence, transmission and recovery rate. Each dot represents the clone's mean value for the first principal component of related traits (see Table 1). Columns of letters indicate the clone's ranking. No value is shown for recovery rate of ER in naïve mice as parasitaemias were still at detectable levels on day 19 p.i.

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Table 3. Across-mouse (phenotypic, above diagonal) and across-clone (genetic, below diagonal) regression estimates from pairwise analyses of the first principal components representing groups of traits

(Regression parameters are the separate slopes for naïve and immunized mice and the difference in the height of the 2 lines at the overall mean value of the regressor trait. For phenotypic regressions, the dependent variable is shown in the column heading and the independent variable is shown in the row heading. The reverse is true for genetic regressions. Asterisks denote whether slopes are significantly different from zero, and whether the heights differ between the treatment groups ***, $P<0{\cdot}001$; **, $P<0{\cdot}01$; *, $P<0{\cdot}05$; †, $P<0{\cdot}10$. Superscripts 'a' and 'b' indicate that the slope in naïve mice differs significantly from the slope in immunized mice at the $P<0{\cdot}05$ and $P<0{\cdot}10$ levels respectively.)

| Trait (first principal component) | Regression parameter | Growth | Virulence | Recovery rate | Transmission |
|---|---|---|---|---|---|
| Growth | Slope in naïve | | 0·80*** | −0·17[b] | 0·37* |
| | Slope in immunized | | 0·81*** | −0·52*** | 0·35** |
| | Difference at mean | | 0·34 | −1·15*** | 1·06*** |
| Virulence | Slope in naïve | 0·68* | | −0·27†[a] | 0·30* |
| | Slope in immunized | 1·13*** | | −0·67*** | 0·26* |
| | Difference at mean | 0·23 | | −0·98** | 1·16*** |
| Recovery rate | Slope in naïve | −0·13[b] | −0·23[b] | | −0·64 |
| | Slope in immunized | −0·90** | −0·82*** | | −0·29** |
| | Difference at mean | −0·69† | −0·60* | | 0·82* |
| Transmission | Slope in naïve | 0·50 | 0·47 | −0·53 | |
| | Slope in immunized | 0·43 | 0·27 | −0·34 | |
| | Difference at mean | 0·89† | 0·93† | 0·81 | |

immunized mice (Fig. 3). However, the heights of the regression lines of virulence on growth were the same in both treatments (Table 3, Fig. 3). Results for individual traits were similar to those for principal components except when virulence was regressed on day 5 parasitaemia when the curves were lower in immunized mice than in naïve mice ($P<0{\cdot}001$ for maximum RBC loss and rate of weight loss, $P<0{\cdot}4$ for maximum weight loss, data not shown).

Unlike other traits, regression slopes involving recovery rate traits differed between immunized mice and naïve mice, being steeper in immunized mice than in naïve mice when recovery rate was the dependent variable (Table 3, Fig. 3). The relationships were negative as expected. As for transmission potential, there was a greater rate of recovery in immunized mice than in naïve mice at the overall average level of virulence and growth indicating that immunization increased recovery rate disproportionately more than it suppressed virulence and growth.

## DISCUSSION

We found that the positive relationships between growth, virulence and transmission potential of *P. chabaudi* infecting naïve mice described in our previous study (Mackinnon & Read, 1999*a*) were maintained under semi-immunity, and that immunity reduced all these fitness-related traits. Immunity also cleared parasites earlier, as shown in previous studies (Jarra & Brown, 1985; Buckling & Read, 2001). These relationships were reflected across parasite clones: genetically more virulent and transmissible clones in naïve mice were generally more virulent and transmissible in immunized mice relative to other clones. Thus, in effect, host immunity rendered a virulent parasite less virulent, with associated reductions in life-time transmission potential. These data support the basic assumption of our theoretical model that predicts that increasing the level of anti-disease defence in the host population, e.g. through vaccination, will drive parasites to evolve higher levels of intrinsic virulence (Gandon *et al*. 2001). As far as we are aware, these data represent the only direct test of this assumption in a pathogen of relevance to human disease.

We also found that the heights of the transmission versus virulence and growth rate regression lines were lower in immunized mice than in naïve mice. Thus, while the magnitude of the relationship was the same in both host types, there was a further unexpected reduction in transmission potential from immunized mice. There are several possible explanations for this observation. First, there may have been a single unifying, but curvilinear, relationship between the traits that encompassed the data from both environments but was undetectable due to limited statistical power. The fact that the regression lines did not join up at the average value of the independent variable despite considerable overlap between them, and the failure to detect curvilinearity within treatments in the vast majority of regression analyses, suggests that this is unlikely. Secondly, the extra reduction in transmission potential may truly reflect an extra component of immunity against transmission stages that is unrelated to growth rate or virulence. We know of no direct experimental

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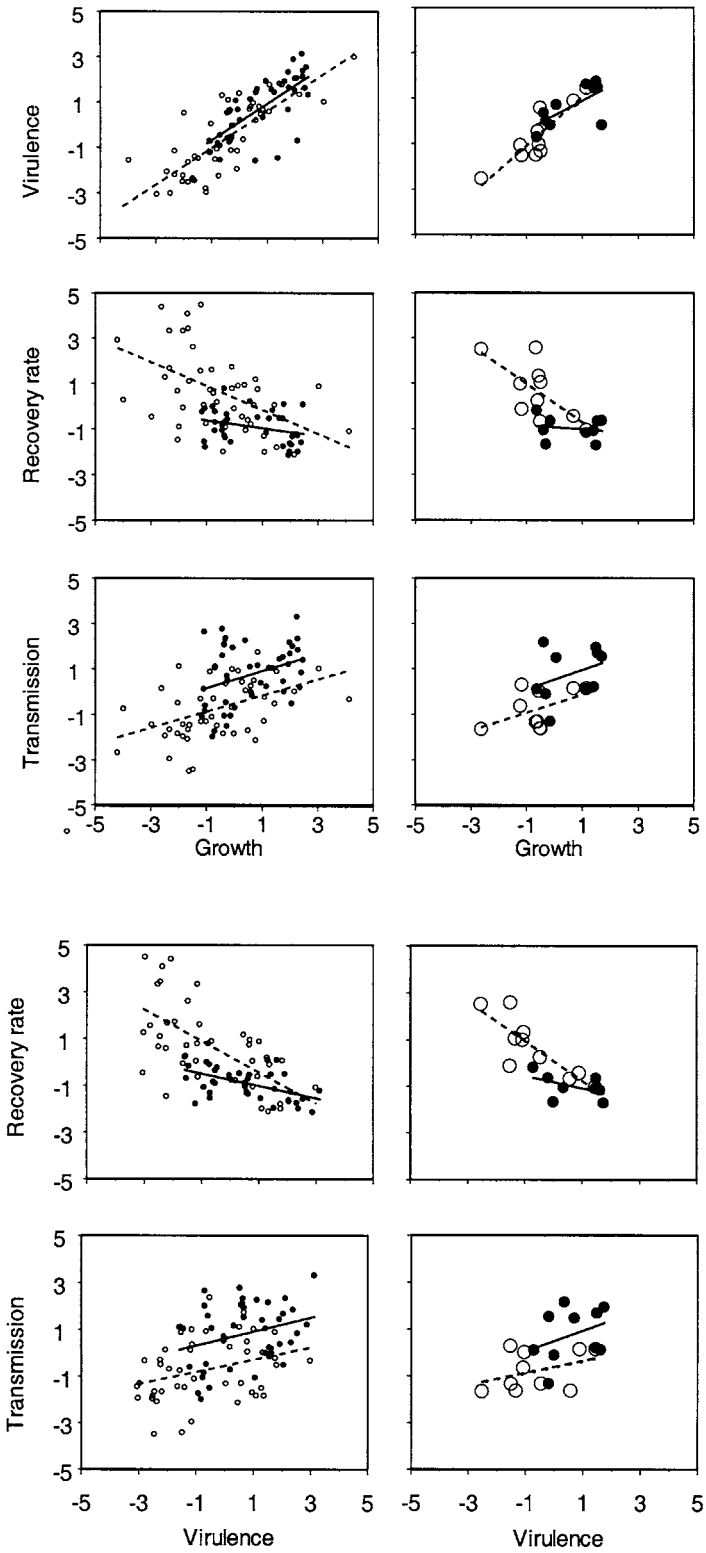


Fig. 3. Phenotypic (across-mouse, left) and genetic (across-clone, right) relationships among traits relating to growth rate, virulence, transmission and recovery rate in *Plasmodium chabaudi* in naïve (solid symbols) and immunized (open symbols) mice. Lines show least-squares best-fit regression lines within naïve (solid lines) and immunized (broken lines) mice.

evidence of immunity that selectively removes gametocytes (Taylor & Read, 1997), but even if it does exist, it is difficult to see how we could have elicited it given that we generated immunity through a short-term infection with parasites of which the vast majority were asexual forms. A more likely explanation is that immunity is more effective in clearing parasites during the period when most gametocytes are produced than when most asexual parasites and maximum virulence are produced, thus decreasing the transmission rate : virulence ratio. Most theoretical models assume that virulence and transmission rate

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are constant throughout the infection which is clearly an oversimplification for malaria and many other diseases. Nevertheless, the extra reduction in transmission potential in immunized hosts does not affect the qualitative outcome of our model as long as the transmission–virulence slope remains the same (Gandon *et al*. 2001), and in fact selects for higher virulence than we predicted when assuming that the heights of the curves were also the same. This example illustrates that conclusions drawn from theory can be robust to at least some complex realities not captured in models.

In this study we immunized with one clone (ER) and, in 9 out of 10 cases, challenged with different clones: thus we were observing the effects of heterologous immunity. In malaria parasites, including *P. chabaudi*, heterologous immunity is weaker than homologous immunity due to strain specificity in the antigenic profile of distinct parasite clones (e.g. Jarra & Brown, 1985). Such a pattern was not obvious for ER in this study but the effects of intrinsic growth rate are likely to have overridden any effect of strain-specific immunity which is weak compared with the effect of immunity in general. Given the apparent importance of strain-specific immunity in disease severity in malaria (e.g. Bull *et al*. 1998), determining whether the virulence–transmissibility relationship we observe here is robust to variation in the degree of cross-immunity is currently a research priority. For example, Buckling & Read (2001) observed strain-specific effects on gametocyte infectivity to mosquitoes but not gametocyte production: whether such interactions are general remains to be determined.

A new feature of this study compared to our previous studies on characterizing *P. chabaudi* infections is that we quantified clearance rate, or length of infection. In many evolutionary models, host recovery rate is included as a key component of fitness and, like transmission rate, is assumed to be biologically related to virulence (Anderson & May, 1982; May & Anderson, 1983; Frank, 1996; Antia & Lipsitch, 1997; Van Baalen, 1998; Ganusov, Bergstrom & Antia, 2002). In the present study, we found lower recovery rate of more virulent and faster growing parasites, even in the absence of host death, but only in semi-immune mice. Thus we have empirically demonstrated another fitness advantage to virulence in addition to that of higher transmission rate, but one that is only detectable in immunized animals. When this virulence-recovery rate trade-off is incorporated into our model of how vaccination drives virulence evolution, the prediction that anti-disease vaccines select for higher virulence still holds (Gandon *et al*. 2001). In this study we did not measure the infection dynamics after day 19 which, typically in *P. chabaudi*, show 1 or 2 more recrudescent peaks due to antigenic variation (reviewed by Phillips *et al*. 1997). However, the contribution of these peaks to overall transmission is likely to be minimal given that parasite densities after day 19 p.i. are of 2–3 orders of magnitude lower than prior to this. Nevertheless, the role of long-term persistence in parasite fitness is an open question in malaria, and one that needs further investigation.

Virulence evolution models also assume that transmission saturates at high levels of virulence, i.e. approaches a horizontal asymptote: otherwise, virulence would evolve to an infinitely high level (Levin & Pimentel, 1981; Anderson & May, 1982; Frank, 1996). In this study, there were only very mild indications of curvilinearity of the saturating kind: this lack of evidence is not surprising given the large amount of between-mouse variation in all traits. Whether transmission saturates with virulence in nature will depend on how gametocyte density relates to the number of mosquitoes that become infected and successfully transmit, about which little is known. We do know from laboratory data that the proportion of mosquitoes infected increases with increasing gametocyte density (reviewed by Taylor & Read, 1997), although infection rates are usually unnaturally high in these artificial environments. Also, we have found evidence of an upper limit to gametocyte production at high virulence levels which was not due to host death in the *P. chabaudi* system (Mackinnon, Gaffney & Read, 2002). Finally, high infection loads in mosquitoes may reduce mosquito survival (Ferguson & Read, 2002*a*) and therefore, perhaps, transmission to new hosts. Thus it seems likely that there are upper limits to transmission rate in the field, as assumed by the evolutionary models.

How might these results relate to *P. falciparum* malaria in the field? In the laboratory, we can only measure correlates of parasite fitness (i.e. morbidity rather than mortality rate, life-time gametocyte production rather than number of new hosts infected per unit time), and these only incompletely. The same, unfortunately, is true of field data. We can therefore only draw parallels between field (*P. falciparum*) and laboratory data (from *P. chabaudi* and other rodent malarias) in supporting the assumptions of our virulence evolution model. This we do now. First of all, there is between-strain variation for virulence and growth rate in *P. falciparum* as measured *in vivo* (James, Nicol & Shute, 1932; Covell & Nicol, 1951; Gravenor, McLean & Kwiatkowski, 1995; Molineaux *et al*. 2001; Simpson *et al*. 2002). *In vitro* evidence also shows that strains vary in growth rate (Chotivanich *et al*. 1998; Simpson *et al*. 1999) and in transmissibility (Graves, Carter & McNeill, 1984). Secondly, virulence is positively related to asexual population size across hosts (e.g. Field & Niven, 1937) and with asexual growth rate at the parasite strain (genetic) level (Gravenor *et al*. 1995; Chotivanich *et al*. 1998; Simpson *et al*. 1999). Thirdly, at least across hosts, gametocyte density increases with asexual parasite density (Molineaux & Gramiccia,

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1980; Nacher *et al*. 2002) and disease severity (Nacher *et al*. 2002). Fourthly, death usually occurs when asexual parasite loads are maximal, and this occurs prior to when the bulk of gametocytes are produced (reviewed by Kitchen, 1949). Fifthly, prior exposure and therefore acquired immunity reduces virulence, asexual population size and gametocyte densities (reviewed by Taliaferro, 1949). All these hold for *P. chabaudi* in laboratory mice (Jarra & Brown, 1985; Taylor *et al*. 1997*a*, *b*, 1998; Mackinnon & Read, 1999*a*, *b*; Buckling & Read, 2001; Timms *et al*. 2001; Mackinnon *et al*. 2002) and thus provide us with a laboratory model for examining broad relationships among parasite life-history components related to virulence. Nevertheless, in both *P. chabaudi* and *P. falciparum*, there are many further aspects of virulence evolution that need investigating such as the costs and benefits of virulence in the parasite's other host, the mosquito (Ferguson & Read, 2002*b*), and the impact of mixed-genotype infections on parasite fitness (Taylor *et al*. 1997*a*, *b*, 1998; Read & Taylor, 2001; Read *et al*. 2002).

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