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The effects of mosquito transmission and population bottlenecking on virulence, multiplication rate and rosetting in rodent malaria

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Abstract

Malaria parasites vary in virulence, but the effects of mosquito transmission on virulence phenotypes have not been systematically analysed. Using six lines of malaria parasite that varied widely in virulence, three of which had been serially blood-stage passaged many times, we found that mosquito transmission led to a general reduction in malaria virulence. Despite that, the between-line variation in virulence remained. Forcing serially passaged lines through extreme population bottlenecks (<5 parasites) reduced virulence in only one of two lines. That reduction was to a level intermediate between that of the virulent parental and avirulent ancestral line. Mosquito transmission did not reverse the increased parasite replication rates that had accrued during serial passage, but it did increase rosetting frequencies. Re-setting of asexual stage genes during the sexual stages of the life cycle, coupled with stochastic sampling of parasites with variable virulence during population bottlenecks, could account for the virulence reductions and increased rosetting induced by mosquito transmission.

Keywords: Malaria; Virulence; Mosquito transmission; Population bottlenecking

1. Introduction

In the laboratory, serial passage rapidly increases the virulence of many microparasites (reviewed in Ebert (1998, 2000)), including malaria parasites (James et al., 1936; Greenberg and Kendrick, 1956; Sergent and Poncet, 1959; Galli and Brambilla, 1967; Hartley, 1969; Dearsly et al., 1990; Mackinnon and Read, 1999b, 2004a). Knowing why this happens in the laboratory, but apparently not in the wild, would help in understanding what evolutionary forces determine the virulence of disease-causing pathogens of humans and animals. In particular, identifying constraints on the rate of pathogen evolution (Ebert, 2000), such as vector transmission or population bottlenecks (Bergstrom et al., 1999; Wahl et al., 2002), would help determine how quickly parasite populations in the field might respond to selection from intervention measures such as drugs and vaccines (Mackinnon, 1997; Gandon et al., 2001).

Experimentally induced malaria infections in humans and in animal models have demonstrated that there is parasite-derived variation in virulence (harm done to the host; reviewed in Mackinnon and Read (2004b). Specific parasite virulence determinants have yet to be identified, although various phenotypes expressed during the asexual stages of the life cycle, and known to be encoded by parasite genes, are correlated with disease severity (Marsh, 1992; Miller et al., 2002). These include rosetting (a cytoadherent property in which red blood cells infected with mature parasites adhere to other red blood cells (Carlson et al., 1990; Rowe et al., 1997)), sequestration (another cytoadherence property where infected cells adhere to endothelial cells (Ho and White, 1999; Newbold et al., 1999)), in vivo and in vitro proliferation rates (Gravenor et al., 1995; Chotivanich et al., 2000; Mackinnon and Read, 2004b), cell selectivity (Simpson et al., 1999; Chotivanich et al., 2000) and invasion pathways (Yoeli et al., 1975; Freeman et al., 1980; Preiser and Jarra, 1998). Some of these virulence-related phenotypes can vary within parasite genotypes (i.e. exhibit clonal variation) due to differential gene expression of members of multi-gene families rather than because of

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sequence variation (Dolan et al., 1990; Phillips et al., 1997; Kyes et al., 2001; Duraisingh et al., 2003). Mosquito transmission affects expression patterns, with many asexual stage genes switched off and sexual stage genes turned on (Hayward et al., 2000; Lasonder et al., 2002; Florens et al., 2003; le Roch et al., 2003), raising the question of how virulence phenotypes are affected by mosquito passage. Most previous reports indicate that mosquito transmission has no effect on the virulence of serially blood-stage passaged malaria parasites (James et al., 1936; Coatney et al., 1961; Walliker et al., 1976; Knowles and Walliker, 1980; Walliker, 1981; Barnwell et al., 1983), although reductions in virulence or changes in virulence-related characteristics following mosquito passage have also been reported (Alger et al., 1971; Walliker et al., 1976; Knowles and Walliker, 1980; Gilks et al., 1990; Mackinnon and Read, 2004a). However, all these studies suffer from lack of replication and contemporaneous comparison, and so the question of whether mosquito transmission affects virulence remains unresolved.

Here, we report the effects of mosquito transmission on virulence of six lines of *Plasmodium chabaudi* that differ in virulence. The virulence differences were found among lines recovered from the wild or were generated by serial blood-stage passage in the laboratory. We show that mosquito transmission reduces virulence, but that the underlying virulence differences between lines are stable to mosquito transmission. Mosquito transmission increased rosetting rates but did not affect multiplication rates.

2. Materials and methods

2.1. General experimental procedures

Female C57Bl/6J mice aged between 4 and 8 weeks were used throughout the experiments and were supplied from various sources (Harlan, UK, Bantam and Kingman, UK, and the University of Edinburgh). They were housed at 21 °C and fed on diet 41B (Harlan, UK) with 0.05% *para*-aminobenzoic acid (PABA) added to their drinking water to assist parasite growth (Jacobs, 1964). Parasite lines were obtained from their natural host *Thamnomys rutilans* from the Central African Republic and stored in liquid nitrogen prior to use in these experiments (Beale et al., 1978).

To initiate blood-stage infections, donor mice were injected with thawed deep-frozen stocks of infected blood diluted in citrate saline (0.85% NaCl, 1.5% trisodium citrate). After these infections became patent (4–12 days), 2–20 μl of blood was taken from the end of the mouse's tail and diluted in media (50% heat-inactivated calf serum, 50% Ringer solution [27 mM KCl, 27 mM $CaCl_2$, 0.15 M NaCl] with 20 units of heparin per millilitre of blood) to give the required concentration for a standard inoculum of 10^4, 10^5 or 10^6 parasites per mouse. All injections were in a volume of 0.1 ml introduced into the intraperitoneal cavity.

For transmission of lines through mosquitoes, 20–50 mosquitoes were allowed to take a blood meal from one to two anaesthetised mice that had been infected 13–16 days previously and had detectable numbers of gametocytes in their blood. Mosquitoes fed on the same parasite line were kept in individual pots at 25 °C and fed on glucose at a concentration of 10% with 0.05% PABA. Fourteen to 17 days after the blood feed (when sporozoites are normally present in the infected mosquito's salivary glands), mosquitoes were allowed to take a blood meal on an uninfected mouse. If infection ensued, the mouse's blood was aliquoted and stored in liquid nitrogen for later use in the experiments reported here. The proportion of mosquitoes infected with malaria were not monitored in these experiments, although infection rates in other experiments performed around this time were typically between 5 and 20%.

All work was carried out in accordance with the Animals (Scientific Procedures) Act 1986 (UK).

2.2. Experimental design

Six lines of parasites were transmitted through *Anopheles stephensi* mosquitoes and then compared for their virulence with their non-transmitted counterpart lines. Three of these lines (AS, AJ and CW-0) had been recently derived from their natural host, the thicket rat, *T. rutilans*, and hence had just undergone mosquito transmission. However, lines ER, CW-A and CW-V had undergone many blood passages since their natural host. ER had been passaged an unknown number of times and on variable days p.i. prior to this experiment, but had been cloned and passaged through mosquitoes relatively recently (Fig. 1). Lines CW-A and CW-V had been passaged on day 12 p.i. for 12 passages during which they had been subjected to selection for high (CW-V) and low (CW-A) virulence on the basis of how much weight loss they caused their mouse host (Mackinnon and Read, 1999b). Despite this divergent selection for virulence, both lines markedly increased their virulence relative to their unpassaged ancestral line, CW-0, and did not themselves differ at the end of this passage experiment (Mackinnon and Read, 1999b). These two lines had undergone a further 11–13 passages on days 5–8 p.i. for routine purposes prior to this experiment. Details of the history of mosquito passage and cloning in these six lines are shown in Fig. 1.

AS and AJ mosquito-transmitted lines were compared for virulence with their non-transmitted counterparts in each of two separate replicate experiments with five mice per group per experiment. ER lines were compared in one experiment using six mice per group. Lines CW-0, CW-A and CW-V were evaluated in two-independent experiments with five mice in each of the six groups. The size of inoculum was 10^4 and 10^6 parasites per mouse for the two replicates of AS and AJ, 10^6 parasites per mouse for ER, and 10^5 parasites per mouse for the CW lines. Virulence was

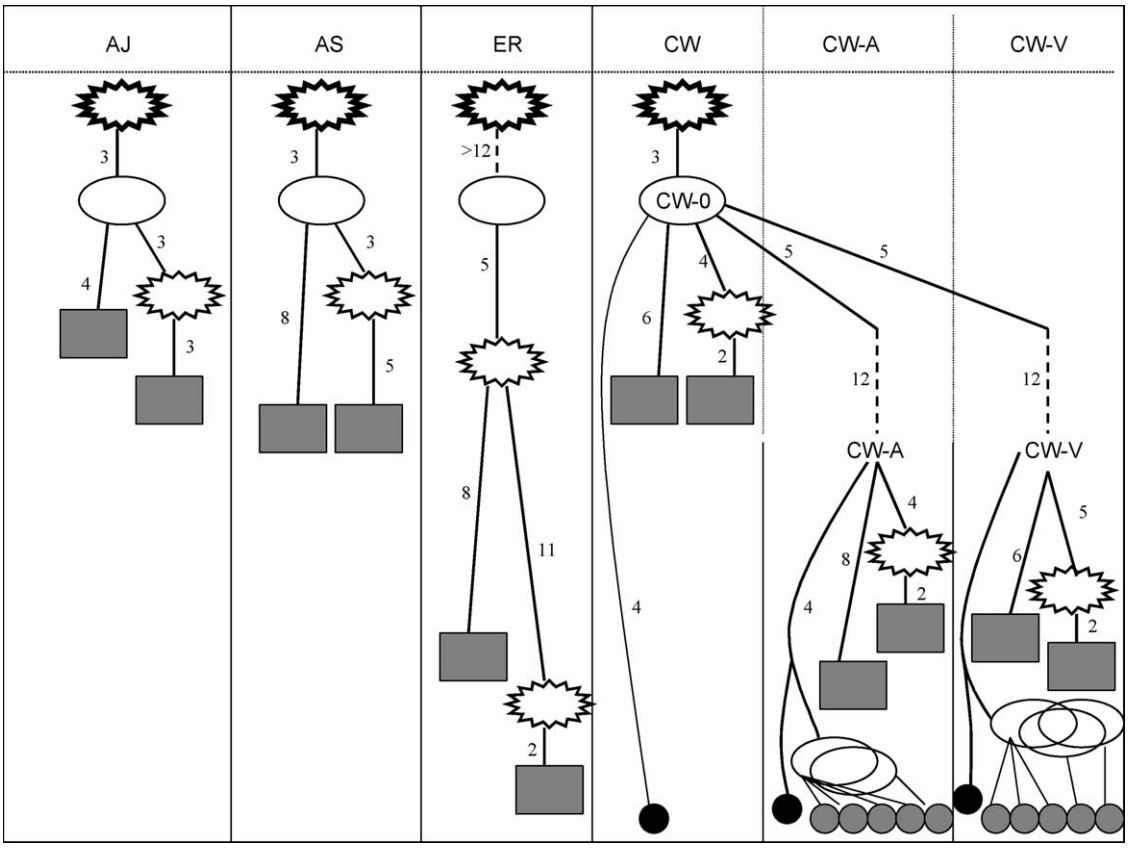

Fig. 1. Passage history of lines of *Plasmodium chabaudi* parasites used in the experiments. Star-shaped ovals indicate that the line was transmitted through mosquitoes; bold edges indicate that this occurred in the parasite's natural environment. Smooth-edged ovals indicate that the line was cloned. Numbers indicate the number of blood-stage passages between these events. Shaded pairs of boxes within each set of vertical lines indicate the pairs of mosquito-transmitted and non-transmitted lines compared in the first set of experiments. Groups of small circles indicate the clones (grey shading) and parent lines (black) that were compared in the second set of experiments.

measured in all the above experiments, but early multiplication rate (see below) was only measured in the second trial of the CW lines. Rosetting (see below) was assessed in the CW lines in two further experiments with three and four mice per line, respectively.

To test the effect of population bottlenecking on virulence, lines CW-A and CW-V were cloned out by limiting dilution. This was done by injecting nine to 16 mice with inocula with an expected number of two or five parasites per injection. Five clones of CW-A were obtained from two separate cloning exercises (Clones 1–4 in the first, Clone 5 in the second) and five clones of CW-V were obtained in three separate cloning exercises (Clones 1–3 in the first, Clone 4 in the second, and Clone 5 in the third). Using inocula of 2–5 parasites per mouse, the number of mice that became infected ranged between 1 out of 10 and 6 out of 15. It is likely therefore that the cloning procedure reduced the population to below five parasites per clone. After storing the clones and parent lines in liquid nitrogen for at least a month, they were compared for virulence in groups of mice (four to five per group) with 10^5 parasites per mouse. This was done in four separate experiments. The uncloned CW-A and CW-V lines, Clones 1 and 2 of each of these lines, and line CW-0 were compared in each of two separate experiments. Lines CW-0, CW-A and Clones 3, 4 and 5 of CW-A were compared in a third experiment, and lines CW-0, CW-V and Clones 3, 4 and 5 of CW-V were compared in a fourth. Thus, each clone was directly compared with its uncloned parent line and the unpassaged ancestral line, CW-0.

2.3. Measurement of virulence, multiplication rate and rosetting

For measurement of virulence, red blood cell (RBC) density was measured every 2–3 days until day 23 p.i. by flow cytometry (Coulter Electronics, Luton, UK), and mouse liveweight was measured every day from day 6 to 13 p.i. and every 2–3 days otherwise. Minimum RBC and liveweight for each mouse were calculated. The difference between these minima and their starting values taken between days 0 and 4 p.i. were calculated to give maximum RBC and liveweight loss. The area between these curves through time and a horizontal line at their starting values was calculated as a measure of total RBC and liveweight loss over the course of the infection.

Rosetting assays were performed as described previously (Mackinnon et al., 2002). Briefly, 5–20 μl of tail blood was taken between 9:00 and 11:00 a.m., cultured overnight, stained with ethidium bromide and then counted the following morning for the number (out of 100) infected cells that were unbound vs. bound to two or more uninfected cells. Between one and five measurements were made on each mouse from the first day of patency until just before peak parasitaemia when agglutinates and lysed cells were commonly seen, thus making counting inaccurate.

Multiplication rate during the early phase of the infection (days 2–7 p.i.) was measured using real-time quantitative PCR. Two microlitres of tail blood was taken from each mouse at around 10:00 a.m. (i.e. when most parasites seen in the peripheral blood were in the ring or early trophozoites stages) and added to 100 μl of citrate saline on ice. Samples were subsequently pelleted by centrifugation, the citrate saline removed and the blood stored at −80 °C until required. DNA extraction was performed using the BloodPrep® kit (Applied Biosystems) on the ABI Prism® 6100 Nucleic Acid PrepStation according to manufacturer's instructions. DNA was eluted in a total volume of 200 μl and stored at −80 °C until quantification. PCR primers and a minor grove-binder (MGB) probe, targeting the *P. chabaudi MSP-1* gene, were designed using Primer Express® software (Applied Biosystems); amplicon length 110 bp. Two microlitres of DNA was included in a 25 μl volume PCR reaction with the following components: 1.5 μl each of forward (5′ CCG GAA GAA CTA CAG AAT ACA CCA T 3′) and reverse primer (5′ TGT AGA GAA GTA GAA AAT ACA GAT AGG GCT AA 3′), both at a final concentration of 300 nM; 12.5 μl of TaqMan® Universal PCR Master Mix (hot start); 1 μl of MGB probe (5′ 6-FAM-ACA AGT ACA TAC AAT TTT T-MGB 3′) at a final concentration of 200 nM and 6.5 μl of sterile water. Amplification was performed on an ABI Prism® 7000 real-time thermalcycler with an initial denaturation of 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Absolute quantification of experimental samples was performed by comparing threshold cycle numbers against a standard curve. DNA standards, covering six orders of magnitude (2–200,000 parasites), were generated from duplicate DNA extractions of blood samples bearing known parasite numbers. Standards were extracted from the same volume of blood and using the same methodology as for experimental samples. Duplicates of each standard (or triplicates for lower template numbers) were included in each quantitative PCR run. Repeatability of the quantification procedure was assessed by performing duplicate assays on 30 of the experimental samples selected at random from across days and parasite lines. The assay was found to not amplify mouse or mosquito DNA.

2.4. Statistical analysis

To test the effects of mosquito passage on virulence, a linear model with fixed effects for treatment (mosquito-transmitted or not), parasite line (AJ, AS, ER, CW-0, CW-A, CW-V), experimental replicate and an interaction between these main effects (where significant, $P<0.05$) was fitted to data on each of the virulence measures described above. To test the effect of cloning on virulence, a model was fitted with fixed effects for parental line (CW-0, CW-A, and CW-V), clone within line and experimental replicate. Multivariate analyses of all virulence traits together were also performed.

For analysis of rosetting data, the same model as for the mosquito transmission experiments was fitted but with inclusion of a random effect for mouse to allow for covariance between repeated measures on the same mouse. Data were analysed on the raw and arcsine square-root transformed scale.

For analysis of multiplication rate data, which were $\log_{10}$ transformed prior to analysis, the model included fixed effects for treatment, parasite line, an interaction between these, a linear covariate for day p.i., interactions between this covariate and the preceding terms, and random effects for mouse and a mouse by day p.i. interaction. Thus, the model allowed the estimation and testing of treatment and line differences in the intercept and slopes of the regression of parasite number through time, while allowing for covariances among repeated measures on the same mouse. A quadratic term for the covariate was also tried in the model to detect a decrease in multiplication rate just prior to peak parasitaemia (day 6–7 p.i.).

F-tests and *t*-tests were used to determine the statistical significance of variation among groups or between pairs of experimental treatment groups. Least-squares (i.e. adjusted for other effects) means of these groups and their 95% confidence limits (based on the residual variance after fitting the model) were calculated.

Relationships among traits (virulence measures, rosetting frequency, and the slope and intercept from multiplication rate data) were described using Pearson correlations. These were computed on the experimental group means ($n=6$) and on the residuals after adjusting for experimental group effects ($n=30$) to determine the relationships across and within groups, respectively.

Repeatability of DNA quantification (i.e. the correlation between repeated measurements on the same sample) was calculated as the ratio of the variance due to mouse (estimated by fitting mouse as a random effect in the model) relative to the total variance remaining after accounting for systematic effects by fitting parasite line, treatment and day p.i. as fixed effects in the model. Likelihood ratio tests were used to determine statistical significance of the mouse effect under the assumption that the test statistic was distributed as a chi-squared variable with one degree of freedom.

3. Results

3.1. Virulence

Mosquito passage reduced the virulence, as measured by minimum RBC density, in all six-parasite lines (Fig. 2). This effect was highly significant when pooled across all lines ($P<0.001$) and was consistent across lines (line by treatment interaction, $P=0.5$). Tests on individual lines were only marginally significant ($P<0.05$ in three out of six cases).

Variation among lines was highly significant both before and after mosquito transmission ($P<0.001$) even when excluding the highly passaged lines, CW-A and CW-V. Differences between CW-0 and the CW-V line remained after mosquito transmission ($P<0.001$) but not between the CW-0 and CW-A line ($P>0.05$, Fig. 2). Thus, clone differences generated by serial passage in the laboratory, or that existed naturally in the wild, were generally stable to mosquito transmission. There was no relationship between the magnitude of the mosquito transmission effect (mean value) and the difference between the number of asexual passages since the last mosquito transmission (Pearson correlation on six values, $r=0.44$, $P=0.4$).

The effects of population bottlenecking on virulence varied between lines. Based on analyses of minimum RBC density, none of the five clones made from the CW-A line differed in virulence from their uncloned parent line ($P=0.16$–0.88) or amongst themselves ($P=0.7$). In contrast, all five clones made from the CW-V line had lower virulence than their uncloned parent line ($P<0.001$ to $P=0.051$) (Fig. 3). These CW-V clones themselves differed in virulence ($P<0.05$), covering the wide range between that of the uncloned parent (CW-V) and the ancestral line, CW-0. Both the uncloned lines and nine of their 10 clones had higher virulence than the ancestral line CW-0 ($P=0.09$ to $P<0.001$). Thus, cloning did not eliminate the virulence that had accrued during serial passage. Experimental replicate effects were significant ($P<0.01$) but replicate by clone interactions were not ($P>0.05$).

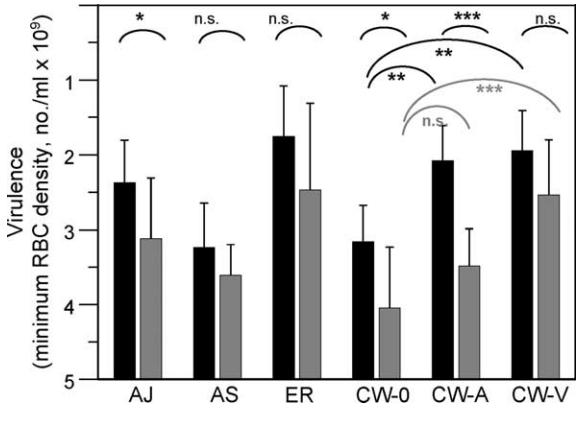

Fig. 2. The effects of mosquito transmission on the virulence, as measured by minimum red blood cell (RBC) density, of six lines of *Plasmodium chabaudi*. Data are the means and upper 95% confidence limits for groups of five to 10 mice infected with lines that had been (grey) or had not been (black) transmitted through mosquitoes. Statistical significance of differences between pairs are indicated (n.s., $P>0.10$; †, $P<0.10$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

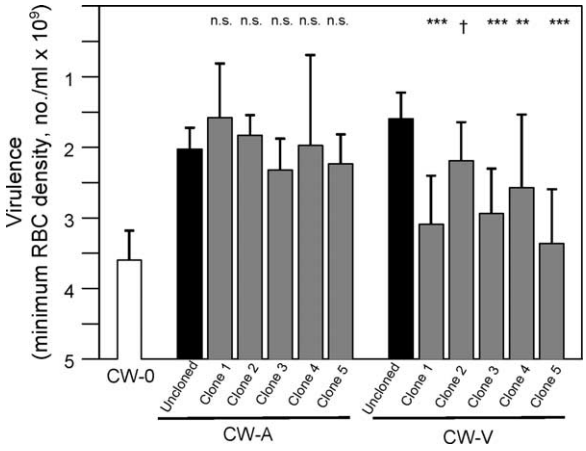

Fig. 3. The effects of cloning on the virulence of lines of *Plasmodium chabaudi* that had been serially passaged under selection for avirulence (CW-A) and virulence (CW-V) from the ancestral line, CW-0. Data are the means and upper 95% confidence limits of minimum red blood cell (RBC) density on groups of mice infected with different lines. Significance of differences between clones and their uncloned parent lines are shown at the top of the figure (see legend of Fig. 2). All lines were significantly different from the unpassaged ancestral line (CW-0, $P<0.01$) except for Clone 1 ($P=0.09$) and Clone 5 ($P=0.75$) of CW-V.

Qualitatively similar results were obtained from univariate analyses of other traits (minimum liveweight, maximum liveweight and RBC loss) and from multivariate analyses of all traits combined (data not shown).

3.2. Multiplication rate and rosetting

The fit of expected values for DNA standards on the log scale against cycle number at the chosen threshold all had R^2 values above 99.2% and slopes averaging -0.28 (range -0.29 to -0.26). This is slightly lower than the expected maximum value of -0.30 corresponding to a doubling of DNA concentration per cycle. Repeatability of DNA quantifications on the same samples were 0.58 within parasite lines and day p.i. ($P<0.05$ by likelihood ratio test), and 0.93 across all lines and days ($P<0.001$ by likelihood ratio test).

Of the 30 mice, DNA was below the detection threshold in five, four and two mice on days 2, 3 and 4 p.i., respectively. Linear regressions for individual mice had R^2 values that averaged 0.93 (range from 0.69 to 0.99). Fitting a quadratic effect for day p.i. did not improve the fit of the model ($P>0.05$). The average slope on the log scale was 0.62 corresponding to a multiplication rate of 4.2 per cycle, and the average intercept was 3.97, corresponding to an initial detectable population size of 9.3×10^3 parasites per millilitre. However, the CW-A and CW-V lines had higher multiplication rates than the CW-0 lines (4.7 vs. 3.4 per

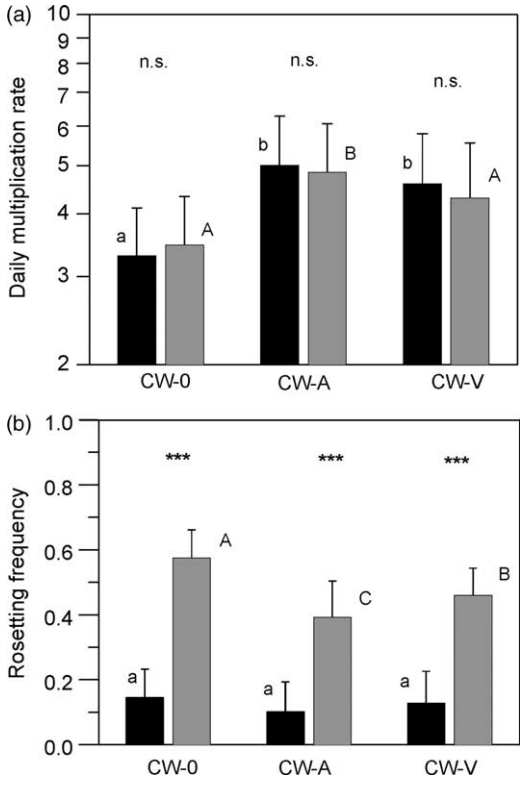

Fig. 4. The effects of mosquito transmission on (a) parasite multiplication rate early in the infection and (b) rosetting frequency in lines of *Plasmodium chabaudi* that had (CW-A and CW-V) or had not (CW-0) undergone serial asexual passage and then had (grey) or had not (black) been transmitted through mosquitoes. Symbols above the pairs of bars indicate statistical significance of mosquito transmission effects (see legend of Fig. 2). Sets of lower and upper case letters indicate statistical differences between CW-0 (labelled with a or A) and the other two lines (b or B, $P<0.05$; c or C, $P<0.01$).

cycle, $P<0.01$ combining CW-A and CW-V which did not differ, $P>0.10$, Fig. 4a).

Mosquito passage significantly increased rosetting frequency in all three lines by an average of 0.36 ($P<0.001$, Fig. 4b). The CW-0 line had significantly higher rosetting than the CW-A and CW-V lines after mosquito transmission ($P<0.05$) though not before ($P>0.10$).

Minimum RBC density was not significantly related to multiplication rate within experimental groups ($r=-0.19$, $P=0.30$, $n=30$) nor across groups ($r=-0.69$, $P=0.13$, $n=6$), nor to rosetting across groups ($r=0.75$, $P=0.09$, $n=6$).

4. Discussion

These experiments showed that mosquito transmission systematically caused a reduction in virulence and an increase in the rosetting of lines of *P. chabaudi*. Importantly, this reduction in virulence did not eliminate the differences among lines that had accrued through serial passage, nor the intrinsic differences that existed in the natural parasite population.

There are several possible explanations for why mosquito transmission reduced virulence and increased rosetting. The first is that the process of meiosis and/or transition through the mosquito stages of the life cycle re-sets the expression of virulence and cytoadherence-determining genes to some lower level. Changes in expression level of almost all of the parasite's genes has been shown to be very tightly regulated through the asexual replication cycle with complete switching on and off each cycle (Bozdech et al., 2003). It seems likely that transcriptional regulation is also highly controlled and specific through the sexual stages (Lasonder et al., 2002; Florens et al., 2003; le Roch et al., 2003). Thus, virulence and cytoadherence-determining genes switched on during blood-stage infections (e.g. the expression of ligands for receptors on the RBC) would be expected to be turned off in the mosquito stages, and different forms may be turned on when the asexual stage of the cycle starts again. Phenotypic switching of antigens and ligands encoded by multi-gene families is a major feature in malaria parasites of various species (Dolan et al., 1990; Phillips et al., 1997; Kyes et al., 2001; Duraisingh et al., 2003) and many of these molecules have been implicated in virulence (Freeman et al., 1980; Gilks et al., 1990; Rowe et al., 1997; Preiser and Jarra, 1998). Such genes are thus prime candidates for mosquito-induced changes in the virulence-related phenotypes observed here.

If such epigenetic control of phenotypes explains the reductions in virulence and increase in rosetting, then the implication from these data is that when virulence-related asexual stage genes are turned back on after mosquito transmission, less virulent and more cytoadherent genes are being expressed first, or come to dominate the population early in the infection. Evidence to support a hierarchy of appearance of the multicopy *var* genes of *Plasmodium falciparum*-genes that have been implicated in virulence through their role in cytoadherence, and which also undergo phenotypic switching-following mosquito transmission has been found in *P. falciparum* (Peters et al., 2002). In *P. chabaudi*, while there are no homologues of *var* (Janssen et al., 2002), there are many multi-gene families (Fischer et al., 2003) and phenotypically switching surface proteins (McLean et al., 1987; Gilks et al., 1990) that are thought to have similar virulence-related roles. To date, studies on the expression pattern of these variable proteins have so far yielded an unclear picture as to whether it is altered by mosquito transmission (McLean et al., 1987; Brannan et al., 1993). What is clear from the current study, however, is that rosetting is restored during mosquito transmission, and is apparently selected against during post-peak serial passage (Fig. 4; Mackinnon and Read, 2004a), suggesting that this property is favoured by selection during the early part of the infection.

An alternative (and not mutually exclusive) explanation to the epigenetic explanation given above for the loss of

virulence following mosquito transmission and sometimes during cloning in this and other studies (Walliker et al., 1976; Knowles and Walliker, 1980) is that parasite infections consist of mixtures of avirulent and virulent variants, and that there is strong sampling variation in the very small sub-populations of parasites that make it through the population bottleneck of mosquito transmission. Typically, less than 10 of the 10^{11} or so parasites circulating in the blood of the human host survive through the mosquito (Tchuinkam et al., 1993). This could lead to consistent reductions in virulence (an increase has never been reported), if the aggregate virulence of the line is some non-linear function of the virulences of the individual variants. Aggregate virulence may be determined by the most virulent variant in the population (Isfan, 1968; Alger et al., 1971; Timms, PhD thesis, University of Edinburgh, 2001; de Roode et al., 2003), or virulence may be enhanced by within-host parasite diversity (Taylor et al., 1998; Timms, PhD thesis, University of Edinburgh, 2001), perhaps as a result of a less efficient primary immune response to genetically diverse clones. Intense bottlenecking from such infections would on average reduce virulence.

Mosquito transmission would also reduce the virulence of infections consisting of virulent and avirulent variants if virulent variants have a competitive advantage within infections but are less transmissible. For instance, parasites with gross mutations, such as deletions of sexual or mosquito-stage genes, could have higher replication rates as a result of having a smaller genome to copy, but may be unable to persist through mosquito transmission (Day et al., 1993). Such variants could arise de novo in vertebrate infections and, because of their within-host selective advantages, induce more disease but then be purged by mosquito transmission. This process of dead-end virulence evolution has been used to explain virulence in polio (Levin and Bull, 1994). It remains an important possibility that some malarial disease could be due to virulent variants arising de novo in the very large parasite populations that make up individual malaria infections. Such a process, repeatedly occurring within infections, could occur on top of intrinsic line differences in virulence, which we have previously shown are positively associated with transmission (Mackinnon and Read, 1999a, 2004b). We note, however, that all serially passaged lines in this and other experiments in our laboratory (Mackinnon and Read, 1999b, 2004a; Ferguson et al., 2004) are readily transmitted through mosquitoes.

The reductions in virulence through cloning in this and other studies (Alger et al., 1971) suggests that stochastic or selective loss of virulent variants could be involved in reductions in virulence following mosquito transmission. However, the fact that all lines appeared to lose virulence during mosquito transmission, while cloning only reduced virulence in some lines, suggests that re-setting of expression levels of virulence determinants during mosquito transmission is also involved.

Our observations of weak or no relationships between virulence, rosetting, and early multiplication rate appear to be contradictory to the body of evidence (albeit mostly circumstantial) in *P. falciparum* malaria that suggests that cytoadherence, asexual multiplication rate and virulence are all positively associated (reviewed in Mackinnon and Read (2004b)). One interpretation of these differences is that these properties are highly dynamic during the course of an infection, and that we were measuring these properties at different points in the infection than those used in studies on human malaria. The fact that parasites with stronger rosetting properties appear at the beginning of an infection post-mosquitoes, but are not selected during post-peak serial passage, may indicate that this property is most favoured at the beginning of an infection. Studies on the fitness advantages and disadvantages of various virulence-related phenotypes at different stages of the infection should provide insight into within-host selective forces operating on virulence.

Our results show that serial passage, which increases virulence in this (James et al., 1936; Greenberg and Kendrick, 1956; Sergent and Poncet, 1959; Galli and Brambilla, 1967; Hartley, 1969; Dearsly et al., 1990; Mackinnon and Read, 1999b, 2004a) and other pathogen systems (Ebert, 1998, 2000), leads to higher parasite multiplication rates, presumably as a consequence of ongoing adaptation to novel hosts. This begs the very important question of what constrains the evolution of virulence in nature. There is some experimental evidence in RNA viruses (which have very high-mutation rates) that virulence can become attenuated during regular bottlenecks due to these stochastic processes (Chao, 1990; Domingo and Holland, 1997; Miralles et al., 2000; Elena et al., 2001). However, transmission bottlenecks are only important in constraining evolution when the effective population size (which depends on the number of hosts that share the parasite population) is small (Bergstrom et al., 1999; Elena et al., 2001), as in the laboratory situation. In the field, the effective size of malaria parasite populations is very large and there is regular genetic exchange among lineages emerging from individual hosts (Anderson et al., 2000). Thus, the mosquito-induced reductions in virulence we report could reduce overall levels of virulence in a population, but will slow rather than halt or reverse the progress of virulence evolution in nature. Moreover, we found that line differences in virulence, including those acquired during serial passage, remained after mosquito transmission (Fig. 2). Thus, mosquito transmission does not negate the challenge of explaining how natural or medically imposed selection acts on such differences (Gandon et al., 2001; Mackinnon and Read, 2004a,b).

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