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Relative clonal proportions over time in mixed-genotype infections of the lizard malaria parasite *Plasmodium mexicanum*

Alice Flynn Ford [1], Jos J. Schall *

Department of Biology, University of Vermont, Burlington, VT 05405, USA

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ABSTRACT

Vertebrate hosts of malaria parasites (*Plasmodium*) often harbour two or more genetically distinct clones of a single species, and interaction among these co-existing clones can play an important role in *Plasmodium* biology. However, how relative clonal proportions vary over time in a host is still poorly known. Experimental mixed-clone infections of the lizard malaria parasite, *Plasmodium mexicanum*, were followed in its natural host, the western fence lizard using microsatellite markers to determine the relative proportions of two to five co-existing clones over time (2–3 months). Results for two markers, and two PCR primer pairs for one of those, matched very closely, supporting the efficacy of the method. Of the 54 infections, 67% displayed stable relative clonal proportions, with the others showing a shift in proportions, usually with one clone outpacing the others. Infections with rapidly increasing or slowly increasing parasitemia were stable, showing that all clones within these infections reproduced at the same rapid or slow rate. Replicate infections containing the same clones did not always reveal the same growth rate, final parasitemia or dominant clone; thus there was no clone effect for these life history measures. The rate of increase in parasitemia was not associated with stable versus unstable relative proportions, but infections with four to five clones were more likely to be unstable than those with two to three clones. This rare look into events in genetically complex *Plasmodium* infections suggests that parasite clones may be interacting in complex and unexpected ways.

1. Introduction

An individual vertebrate organism (mammal, bird or squamate reptile) may be infected with parasites of two or more species of *Plasmodium*, or multiple genetically distinct clones of a single parasite species (Anderson et al., 2000; Mayxay et al., 2004; Vardo and Schall, 2007; Havryliuk and Ferreira, 2009). Researchers for many years have suspected that interaction both among species and among conspecific clones could play a significant role in the biology of *Plasmodium* (Wenyon, 1926; Richie, 1988). Recent theoretical studies and empirical evidence highlight the importance of clonal interaction within individual hosts for the ecology and evolution of malaria parasites. These results include cross-species cycling in dominance within an infection (Bruce et al., 2000; Bruce and Day, 2003), as well as intraspecific clonal competition (de Roode et al., 2003, 2005a; Wargo et al., 2007), infection virulence (Read and Taylor, 2001; Mackinnon and Read, 2004; de Roode et al., 2005b; Bell et al., 2006; Vardo-Zalik and Schall, 2009), life history traits including the parasite's rate of replication and gametocyte sex ratio (Schall, 2009; Vardo-Zalik and Schall, 2009) and transmission success into the vector (Taylor et al., 1997; Vardo-Zalik, 2009). Lacking in these studies on single-species infections, however, is detailed information on the relative proportions of clones in genetically complex infections and the changes in these relative proportions over time.

For single-species *Plasmodium* infections, information on the relative abundance of clones and the changes in relative abundance over time will certainly offer critical insights into the infection dynamics of malaria parasites. Most interesting for studies of relative abundance of clones in complex infections would be the possible nature of interaction among genotypes, ranging from competition to co-operation, and how clonal interaction influences life history traits. For example, sex ratio theory predicts that both the number of co-existing clones and their relative proportions will drive the ratio of male to female gametocyte cells that is optimal for the fitness of the parasite (Reece et al., 2008). If clonal proportions commonly shift over time in infection, this would present a formidable challenge to the parasite (and to researchers attempting to test theory!). Studies on the relative proportions of co-existing parasite species can use microscopic examination of blood films (Bruce et al., 2000), but determining the relative proportions of conspecific genetic clones has been possible only with recent development of powerful new tools of molecular biology (Wargo et al.,

* Corresponding author. Tel.: +1 802 656 0448; fax: +1 802 656 2914.
E-mail address: jschall@zoo.uvm.edu (J.J. Schall).
[1] Present address: School of Medicine, University of Pennsylvania, Stemmler Hall, Box 47, PA 19104-6087, USA.

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2007; Farnert, 2008; Reece et al., 2008; Ford et al., in press). Thus, after decades of speculation on events taking place within mixed-clone infections of *Plasmodium*, these new techniques allow detailed study of the dynamics of clones over time.

Here we examine the relative proportions of co-existing clones of *Plasmodium mexicanum*, a parasite of western fence lizards, *Sceloporus occidentalis*, in experimentally-induced infections with known identity and number of clones. Relative proportions were followed over time by monitoring microsatellite markers (Havryliuk et al., 2008; Liu et al., 2008; Vardo-Zalik et al., 2009; Ford et al., in press). We sought evidence for three trends. First, we expected to see a change in the relative abundance of the clones over the course of the infections. This expectation was based on findings that co-infecting *Plasmodium* clones compete for resources (de Roode et al., 2003) or drive parasitemia higher (Vardo-Zalik and Schall, 2009), and that mixed-species infections reveal cycling of the relative proportions of parasite species (Bruce et al., 2000). Mixed-clone infections of *Plasmodium falciparum* also reveal cycling in the clones that are present in peripheral blood, with other co-existing clones likely to be sequestered (Farnert, 2008). This suggests there is some complex strategy in place, perhaps even co-operation among clones. Second, we expected more significant fluctuations in relative proportions of clones in genetically more complex infections due to a greater opportunity for intense competition for resources, interaction with the immune system, and/or the greater likelihood of co-existence of clones with different competitive abilities. We therefore compared experimental infections that contained two to five clones. Third, whatever the number of clones, more significant shifts in relative abundance should be associated with higher parasitemia growth rates driven by either competition or co-operation. We tested this hypothesis by quantifying both parasitemia and relative clonal proportions over the history of each infection.

2. Materials and methods

2.1. Study site

The field study was conducted at the University of California Hopland Research and Extension Center, near the town of Hopland, approximately 160 km north of San Francisco, USA. The biology of *P. mexicanum* in its hosts, the western fence lizard, *S. occidentalis*, and two species of sandfly vectors, *Lutzomyia vexator* and *Lutzomyia stewarti*, has been studied at the site for the past three decades (Schall, 1996, 2002; Vardo-Zalik and Schall, 2008, 2009; Neal and Schall, 2010). Lizards were collected by noosing using a fishing pole and fishing line. Drops of blood taken from a toe clip were used to make a thin blood film for each lizard and to store several dried and frozen drops for later genetic analysis. The smear was treated with Giemsa stain, and examined under magnification (1000×) to identify lizards infected with *P. mexicanum*.

2.2. Genotyping infections to detect clones

We extracted DNA from dried blood samples from both the naturally infected donor lizards and experimentally infected recipients (Section 2.3) using the DNeasy kit (Qiangen, Valencia, CA, USA) and the provided protocol. Parasites from donor and recipient lizards were genotyped for two microsatellite markers, Pmx306 and Pmx747, using primers and PCR methods and conditions reported by Schall and Vardo (2007) and Ford et al. (in press). For some experimental infections (Section 2.3) a second set of primers for Pmx306 amplified a shorter DNA fragment; this marker is called here Pmx306s. One primer in the PCR was fluorescently labelled, and the product run on an ABI (Foster City, CA, USA) genetic analyzer instrument which allowed visualisation of microsatellite alleles by length. The data were visualised and analysed using GeneMapper 3.5 software (ABI) which produces a graph (pherogram) that shows the size of each allele (its length based on the number of the included ATT repeat motif for the two markers) (Selkoe and Toonen, 2006). *Plasmodium* is haploid in the vertebrate host, so each size allele seen as a peak on the pherogram represents a distinct clone of the parasite.

2.3. Experimentally infected fence lizards

Two studies, referred to here as Study I (Vardo-Zalik and Schall, 2008) and Study II (Osgood and Schall, 2004), initiated experimental infections by injecting blood from a naturally infected lizard mixed with PBS into non-infected recipient lizards. Donor infected lizards were collected from different locations located across the Hopland field site, thus increasing the likelihood of using distinct clones from sites where the parasite is known to be genetically differentiated (Fricke et al., 2010).

Detailed methods for initiating infections are given in Vardo-Zalik and Schall (2008) and Osgood and Schall (2004). In brief, each lizard was inoculated with an equal number of each clone, for a total of 2×10^8 asexual parasite cells (meronts). Recipient lizards were collected from sites where the parasite has been absent or very rare over the past decades. A comparison of results from scanning smears and a PCR assay revealed that false negatives are very rare using the scanning technique (Perkins et al., 1998). Also, no novel parasite clones (not known from the donor lizards) were detected in the recipients, supporting the assumption that recipient lizards were not infected at the initiation of the experiments. A total of 54 infections were selected from the two experiments for use in this study, chosen based on the number of clones present and the number of samples taken over the experiments' duration. Our study had the disadvantage that the two studies were originally designed for other goals and blood samples were not uniformly taken for every infection. However, using two studies performed by different researchers several years apart has the power of detecting consistent patterns in the biology of genetically complex *P. mexicanum* infections.

Study I provided 33 infections; these were sampled every 10 days after parasites were first seen in the blood (Vardo-Zalik and Schall, 2008, 2009); blood was taken and stored and a thin blood smear made (five samples for most infections). Experimental multiclone infections were produced by mixing the blood of five donor lizards. Donor infections were genotyped at the two microsatellite markers, Pmx306 and Pmx747. Four donors revealed a single allele at both markers, and one donor showed two alleles at Pmx306 and one at Pmx747. Allele sizes for each marker for the five donor lizards are given in Table 1, and mixtures of clones and the replicate number (number of lizards given the same combination of clones) are shown in Table 2. Because parasitemia counts

Table 1
Genotypes (clones) of *Plasmodium mexicanum* used to initiate experimental infections in Study I. Five donor western fence lizards, *Sceloporus occidentalis*, were used (A through E), and the allele size for two microsatellite markers is given. Four donors were single-clone based on two markers, and one was two clone based on one marker.

Donor infection	Marker and alleles	
	Pmx306	Pmx747
A	171	176
B	195	161
C	183	176
D	174, 186	173
E	183	176

Table 2
Experimental infections of *Plasmodium mexicanum* initiated in the parasite's natural host, the western fence lizard *Sceloporus occidentalis* from Study I. Mixtures of blood from donor infected lizards (A–E given in Table 1) were used to initiate replicate infections that harboured two to five clones (number of alleles for two microsatellite markers given). Each infection was scored as "stable" or "unstable" proportions of each clone over time based on the criteria given in Section 2.5 Given is the number of infections in each treatment that were stable and unstable and the range in growth rate of parasitemia (r = slope of growth curve).

	N	Number of alleles			
		Pmx306	Pmx747	*n* Stable proportions (range *r*)	*n* Unstable proportions (range *r*)
Donor mix					
AB	4	2	2	3 (0.03–0.07)	1 (0.001)
D	2	2	1	2 (0.003–0.01)	0
CD	6	3	2	6 (0.02–0.13)	0
CDE	11	3	2	10 (0.01–0.14)	1 (0.10)
ABCD	10	5	3	5 (0.001–0.08)	5 (0.02–0.08)

were available for each infection from each day that blood samples were taken, it was possible to estimate the growth rate of each infection by plotting the natural logarithm of the number of parasites seen in 1000 host erythrocytes on each day versus the number of days that had passed since the first sampling of parasites in the blood. The slope of the linear regression model was taken as the rate of increase of the infection.

Study II provided 21 infections which were sampled weekly beginning when parasites first appeared in the blood and thereafter for 8–13 weeks during the summer of 2001 (Osgood and Schall, 2004). The number and temporal spacing of additional sample times at which each infection was examined varied based on the availability of blood. Each infection was genotyped and analszed at either Pmx306 or Pmx747. A total of 15 donor lizards were used to initiate infections with two, three, four, or five clones. Allele sizes and the number of replicates for each combination are given in Table 3. Parasitemia counts were available for only some of these infections, and only for a few sampling dates.

2.4. Genotyping infections to determine relative proportions of clones

We determined the proportions of the parasite clones by examination of the peak heights on the pherograms. Relative proportions of clones were determined by summing the pherogram peak heights for all alleles detected in the infection, and calculating the proportion of the peak height for each clone. Peak height on a pherogram will be a product of the true proportions of the parasite clones as well as the vagaries of the PCR and genetic analysis instrument. For example, some alleles (length of the microsatellite repeat) may amplify more efficiently in the PCR. Using simulated mixed-clone infections generated by mixing DNA in known concentrations, Vardo-Zalik et al. (2009) and Ford et al. (in press) examined these sources of error using *P. mexicanum* and the microsatellite markers used here. Despite possible sources of bias in the procedure, these studies showed that pherogram peak heights give a good measure of true clonal proportions, and provided an empirically determined confidence interval of proportion measures. These confidence intervals were used to score infections as "stable proportions over time" or "changing proportions over time" (Section 2.5). As an independent check on the reliability of the procedure, we compared the results for the two markers, and the two primer pairs for Pmx306 that gave different DNA fragment lengths. If all of the possible sources of error were negligible, then results should have been very similar for both markers and both primers for Pmx306.

Table 3
Experimental infections of *Plasmodium mexicanum* initiated in the parasite's natural host, the western fence lizard *Sceloporus occidentalis* from Study II. Mixtures of blood from 15 donor single-clone lizards were used to initiate replicate infections that harboured two to five clones (number of alleles for one of two microsatellite markers given). Allele size is given for the mixtures. The same allele size for a specific marker indicates lizards received blood from the same donor. Each infection was scored as "stable" or "unstable" proportions of each clone over time based on criteria given in Section 2.5. Given is the number of infections in each treatment that were stable and unstable.

Locus	*n*	*n* Alleles	*n* Stable	*n* Unstable	Alleles
Pmx 747	3	2	0	3	176, 194
Pmx 747	2	2	2	0	188, 200
Pmx 747	1	3	0	1	176, 191, 212
Pmx 747	1	4	1	0	173, 176, 191, 212
Pmx 306	1	2	0	1	174, 178
Pmx 306	1	3	0	1	171, 174, 198
Pmx 306	1	3	1	0	171, 213, 216
Pmx 306	2	3	2	0	174, 192, 195
Pmx 306	2	4	1	1	174, 195, 207, 216
Pmx 306	1	4	0	1	174, 192, 210, 216
Pmx 306	1	4	0	1	171, 192, 210, 216
Pmx 306	5	5	3	2	171, 174, 198, 213, 216

2.5. Analysis of clonal complexity and stability in proportions of clones

Each infection was scored as low clonal complexity (two to three clones) or high complexity (four to five clones). These criteria was chosen based on previous studies that suggest an important life history trait, virulence, differs between these two groups of infections (Vardo-Zalik and Schall, 2008). The growth rate of infections from Study I was evaluated based on the median rate of increase; slowly growing infections were those with a rate below the median, and rapidly growing infections were those growing more rapidly than the median. In scoring the stability of infections, we used the 90% confidence interval determined by Ford et al. (in press) for peak height ratios observed for mixed-clone *P. mexicanum* infections derived from empirical data. This guideline leads to a margin of error of 0.11 on either side of an observed clonal proportion, and therefore, the genetic composition of an infection was classified as "changing," and therefore unstable if, for any two sample times, the proportions differed beyond the confidence interval.

3. Results

3.1. Comparison among markers

Thirty-two experimental infections from Study I were genotyped for Pmx306, Pmx306s and Pmx747, and one infection for only Pmx306 and Pmx306s. Results for two markers and two primer pairs for Pmx306 were very similar, as would be expected if the method presented a true measure of the relative proportions of clones in infections. For 139 infections/dates that coincided for Pmx306 and Pmx306s, results were very similar except for a single date on which one allele in a five-clone infection failed to amplify. Comparisons for 116 infections/dates between Pmx306 and/or Pmx306s and Pmx747 were less clear because in most infections more alleles were detected in the Pmx306 makers than for Pmx747. That is, two or more clones carried the same allele for Pmx747 (Tables 1 and 2). Nonetheless, for four infections, two alleles were detected for all markers and results matched closely for all 15 dates, and results were consistent for all other dates.

Fig. 1 presents two infections with results for the three markers that show concordance of results. All infections for Study II were genotyped for a single marker, so a similar comparison was not possible for those infections.

All alleles known for the donor infections were detected in all 54 experimental infections. Although the experimental design of

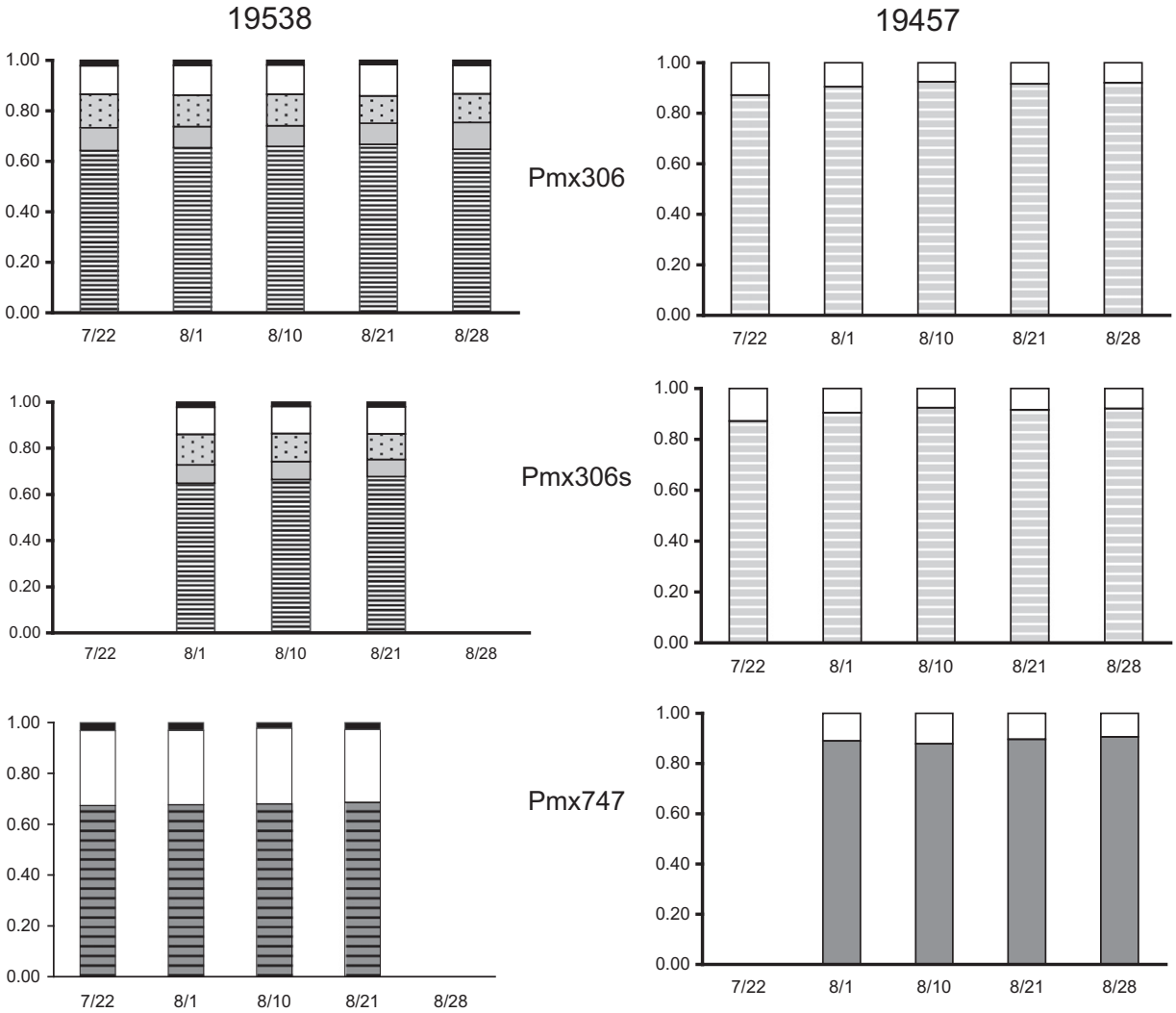

Fig. 1. Two fence lizards experimentally infected with mixed-clone infections of *Plasmodium mexicanum*. Two microsatellite markers (Pmx306 and Pmx747) and two primer pairs for one (Pmx306 and Pmx306s which amplifies a shorter DNA fragment) were used to determine the relative proportions of co-existing clones (*y* axis on each graph) over the course of the infection (dates on *x* axis given as month/day). Lizard 19457 harboured two clones as scored by the two markers, whereas lizard 19538 was infected with five clones (Pmx306), but three of these had the same allele length for Pmx747. Relative proportions measured are nearly identical for Pmx306, Pmx306s and Pmx747 (with three alleles merged for lizard 19538, Pmx747).

both Study I and Study II attempted to introduce equal proportions of each clone into each recipient lizard host, pherogram peak heights revealed that clonal proportions differed on the first sample date for almost all infections (Figs. 1–3).

3.2. Rate of parasite population increase and final parasitemia

Parasite populations varied in their rate of increase over time in the experimental infections, with some remaining almost constant after first becoming patent in the blood, others rising slowly, and still others rising rapidly (Table 2; rates of increase for Study I = −0.031–0.131). The median growth rate used to score infections as rapidly rising versus slowly rising was 0.027. The last parasitemia recorded in the samples also varied substantially (Study I = 3–432; Study II = 7–897). The growth rate for infections in Study I differed among the treatments (Kruskal–Wallis (K–W) test, $P = 0.006$). However, this difference was driven by the higher rate of increase for the mixture from donors C, D and E; removing these lizards resulted in no difference among the other treatments (K–W test, $P = 0.142$). The same clones found in the donors C, D and E were introduced into other treatments, so the effect does not appear to be driven by clone, but by the number of clones or their interaction.

3.3. Relative proportions of clones over time

Combining the infections from both Study I and Study II, we scored 36 (67%) infections as stable, using the criterion of no difference in proportion of any allele between even two dates during the course of the infection, with 18 infections showing a shift for any allele's proportion (Tables 2 and 3). One of the infections scored as unstable had poor amplification of a single allele on a single date (noted above). Stable infections were more common in Study I (79% of 33) than Study II (48% of 21) (*G-test*, $G = 5.57$, $P < 0.05$). Those infections in which clonal proportions changed over time did not demonstrate the cycling of dominance in the peripheral blood seen among mixed-species infections (Bruce and Day, 2003) or among clones in *P. falciparum* infections (Farnert, 2008). Most common was a steady decline or increase in the relative proportion of a single or two clones relative to others present in the host. Examples of infections with stable and changing relative proportions of clones are shown in Fig. 2. Results are shown by date

from the date when parasites were first seen in the blood. Date was chosen rather than days post-patent in the blood to determine whether there was any seasonal effect (both donor and recipient lizards were taken from the wild and recipients kept in cages out-of-doors). The parasitemia for each date is also shown in Fig. 2, showing every combination of stable versus unstable, and rapidly growing versus slowly growing infections (Table 2). No association was detected between stable versus unstable infections

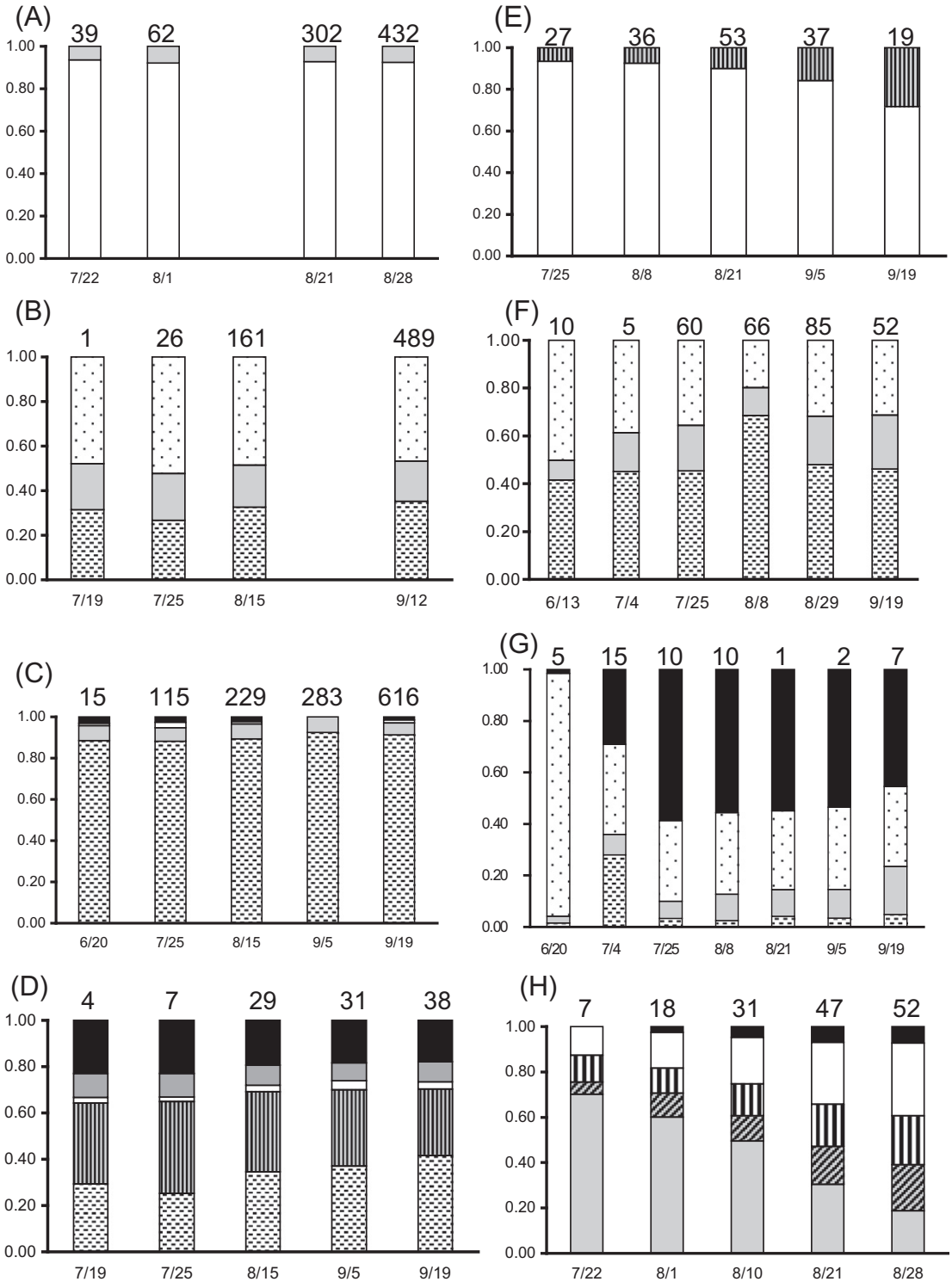

Fig. 2. Relative proportions of clones in eight mixed-genotype experimental infections of *Plasmodium mexicanum* in fence lizard hosts over the course of the infections. (Relative proportions are shown on the *y* axis and dates as month/day on *x* axis for each graph). Each panel presents results for the infection probed for a single microsatellite marker. Four infections (A, B, C, D) are those with stable relative proportions of clones, and four others (E, F, G, H) are those with changing relative proportions. The numbers of clones introduced and established were two (A and E), three (B and F), four (C and G) and five (D and H). Numbers above each bar show the total parasitemia (per 1000 erythrocytes) on that date of the infection. Note that all possible combinations of stable versus unstable and rapidly increasing parasitemia versus slowly growing parasitemia are shown.

Table 4
Number of infections revealing stable versus unstable proportions of clones over time in experimental infections of *Plasmodium mexicanum* in fence lizard hosts (criteria in Section 2.5). Infections are portioned by the number of co-existing clones, from two to five. Combining data into two groups, two and three clones versus four and five clones, to increase sample sizes for a contingency table analysis, the higher number of clones was associated with a greater proportion of unstable infections (χ^2 test, $P = 0.046$).

Number of co-existing clones	Stable infections	Unstable infections
2	7	5
3	19	3
4	2	3
5	8	7

and rate of increase in parasitemia with 12 stable and four unstable 'slow' infections, and 13 stable and four unstable 'rapid' infections ((χ^2 test, $P > 0.05$)). Both stable and unstable relative proportions were seen in infections across clonal numbers, but more complex infections were more likely to be unstable (Table 4). Less than a quarter of infections with two or three clones were scored as unstable, but half of the infections with four or five clones were unstable.

Infections characterised by both stable relative proportions of clones and rising parasitemia indicate that all clones in the infection were increasing at the same rate.

Thus, stable proportions seen in the slowly growing infections represented co-existing clones all growing at the same low rate, and stable proportions in the infections with rapidly increasing parasitemia showed that clones all increased at that same rapid rate. The design of both studies introduced the same clone of parasite (from a single donor) into multiple recipients (Tables 2 and 3), thus allowing examination for a specific clone (genotype) effect on both growth rate and relative proportion of clones. We found that the same clones of parasite were found in both rapidly growing and slowly growing infections, and in infections with unstable relative proportions of clones, the same clone was found to increase in relative proportion in some infections and decrease in others (Tables 2 and 3 and examples shown in Fig. 3).

4. Discussion

The relative proportions of *P. mexicanum* clones were followed over time in mixed-genotype infections that harboured two to five clones (determined by two microsatellite markers). The method had been previously verified (Ford et al., in press), and we confirmed the efficacy of the technique by comparing results for the two microsatellite markers and for two primer pairs for one of those markers (which amplified different lengths of the genome). Results coincided closely for all markers and primer pairs; that is, relative proportions of clones were very similar for all markers. Also, our examination of induced infections produced in two experiments, set several years apart, and conducted by two teams of researchers, came to the same results, indicating the robustness of conclusions.

In both experiments, the goal was to introduce equal numbers of each clone (asexually replicating meronts cells) into recipient lizards. Pherogram peak heights, however, differed significantly the first time parasites were seen for almost all infections. Several causes could have accounted for this result. Counts of asexual cells on the blood smears did not differentiate between meronts with a single nucleus or those with several nuclei, and overall parasitemia and blood cell density counts must always have some error. Thus, equal numbers of parasites may not have been introduced into each lizard. The introduced clones may well have differed in their ability to enter and establish in a lizard (the process of entry of parasites from the body cavity is not normally used by the parasite), or

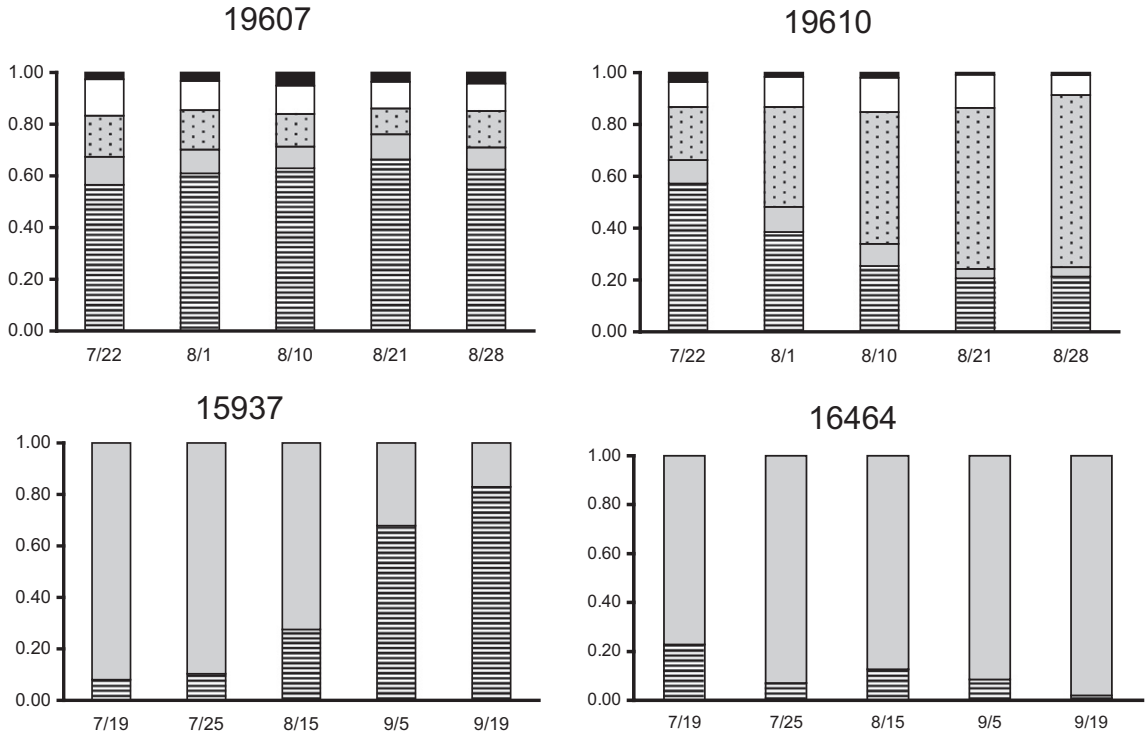

Fig. 3. Four experimental infections of *Plasmodium mexicanum* in the fence lizard host, showing relative proportions of clones (shown on the *y* axis; based on a microsatellite marker) over the course of the infections (dates shown as month/day on *x* axis). Lizards 19607 and 19610 were infected by the same five clones, and 16937 and 16464 by the same two clones; in both cases, the replicate infections were started at the same time from the same mixture of infected blood. For each pair of infections, each microsatellite allele is indicated by the same fill pattern in the graphs for both infections.

clones may have differed in their competitive ability in the early, prepatent period.

Several patterns were observed in the mixed-clone infections. First, over the course of infections, for periods up to 3 months, fully two-thirds revealed constant relative proportions of the co-existing clones. These infections included those with a rapidly increasing parasitemia, in some cases rising to more than half of the erythrocytes infected. This indicates that all clones in such infections were growing at the same rate. The same pattern was seen in slowly growing infections, again indicating that all clones in these infections were growing at the same low rate. Second, for the third of infections with changing relative proportions of clones, there was no obvious cycling of clones. The most common life history was a gradual increase in the relative proportion of one clone, indicating its more rapid growth. Third, there was no association of rate of increase in parasitemia (parasite reproductive rate) with stable versus unstable relative proportions of clones. Last, increased genetic diversity in an infection was associated with a higher probability of unstable relative clonal proportions over time. Infections containing four or five measured alleles at the markers were more likely to display significant shifts in relative proportions of clones (greater than the previously determined measurement error confidence interval) than infections composed of two or three clones. This last trend, however, could be spurious. The number of replicate infections with a specific combination of clones was not equal for all treatments and this could have biased the comparison between stability of clonal proportions with the complexity of the infection. For example, 10 of 19 stable infections containing three clones were replicates with the same combination of clones in Study I (infections started from donors C, D, and E).

These observations could be explained if most parasite genotypes cycling in the lizard host population have the same reproductive rate, with a few clones in the lizard population reproducing at a higher rate. Mixed-clone infections would therefore be most likely to include only the common clones. Variation in overall reproductive rate could be driven by a host effect, with some hosts allowing a more rapid growth rate for all of the clones, and other hosts holding all parasite clones to some lower rate of replication. More complex infections, those with four or five clones, would be more likely by chance to include one or more of the uncommon parasite clones with a higher growth rate. Thus, most infections would reveal stable clonal proportions (with rapid or slow growth rates depending on host quality), and the more complex infections are more likely to include the uncommon parasite genotypes.

We can discount this pleasing scenario. The experimental design of both studies produced replicate infections with the same mixture of clones. We found that these replicate infections varied in overall parasite reproductive rate as expected if host quality indeed determines the replication rate of the parasite clones. However, some replicates with the same co-existing clones showed stable clonal proportions and others revealed changing proportions, and different clones in the unstable infections were the dominant parasite (Tables 2 and 3; examples in Fig. 3). Thus, a clone effect was not observed. Vardo-Zalik and Schall (2009) also found that single-clone infections of *P. mexicanum* all grew over time at similarly low rates, but any combination of these same clones produced infections that often grew at accelerated rates. Therefore, in that experiment there were no "fast" versus "slow" growing genotypes. Combining the findings of Vardo-Zalik and Schall (2009) and our study, a general pattern emerges: when a single clone of *P. mexicanum* is present in a lizard host, infections increase in parasitemia within a fairly narrow but slow range. In mixed-clone infections, approximately half of the infections continued to grow within this narrow range, and half at much higher rates of replication, and reached high parasitemia. In most of the mixed-clone infections, even those with rapid growth, all clones reproduced at the same rate within an infection. In a third of the mixed-clone infections, primarily those with many clones (either slow or rapidly growing), one or more clones outpaced the other clones, leading to unstable relative proportions. Infections with *P. mexicanum* usually last the life of the lizard (Eisen, 2001), and mixed-clone infections are common (from 50% to 80% of infected lizards depending on the site; Vardo and Schall, 2007). Therefore, it would be intriguing to know how clonal proportions vary over time in natural infections of *P. mexicanum* in free-ranging lizards to determine if the trends seen in experimental infections hold in nature.

Three general conceptual views may cast light on the puzzling patterns seen in the experimental infections. Game theory (Maynard Smith, 1982; Nowak, 2006) predicts how players will reach an equilibrium strategy when each player's behaviour depends on the behaviour of other players. The literature on this subject centers on simple games, with two or three players. Simple *Plasmodium* games (two or three clones) may more readily reach some equilibrium characterised by stable clonal proportion —that is, all clones adopt the same growth rate in a co-operative strategy. However, the addition of more players to the game may encourage or facilitate the abandonment of this strategy by one or more outlier players. Chaos theory in biology (Judson, 1994) offers an alternative perspective and notes that most biological processes, including interactions between individuals or species such as competition, are non-linear in structure and thus prone to complex patterns of change. These patterns of change would be difficult or even impossible to explain. A last possible explanation is strictly mechanistic. Hosts certainly vary in quality, including immune system function. Lizards could simply differ in their response to mixed-clone infections which would then lead to a greater chance of instability in the co-existing clones in genetically complex infections. That is, there may be no direct interaction between clones, but instead differences in their responses to the immune attack.

The immune systems of reptiles and mammals must have important functional differences, and thus how clones of malaria parasites interact (if they do at all) in mammals may well not resemble the patterns shown for *P. mexicanum* in fence lizards.

Comparative data, therefore, would be very interesting. The *Plasmodium chabaudi*-rodent malaria model, using laboratory strains in mice, allows quantitative PCR (Cheesman et al., 2003; Wargo et al., 2007), but the method is not feasible for natural systems. This method requires knowledge of variation in gene sequences for design of clone-specific primers. The number of *Plasmodium* clones cycling at local sites can be large (Vardo and Schall, 2007; Farnert, 2008). Several cell surface protein genes in *Plasmodium* vary both by sequence and length, allowing assessment of the numbers of clones within infections. This method has shown that *P. falciparum* clones appear and vanish from the peripheral blood over only hours, most likely because clones differ in their schedule of sequestration in capillaries (Farnert, 2008). This method, however, does not allow measurement of relative proportions of clones in the overall infection. The microsatellite markers offer a more tractable method. The use of microsatellite markers to measure clonal proportions is possible for human malaria parasites (Ford et al., in press) and thus similar data could be gathered under field conditions during public health studies. Thus, as molecular techniques develop and ways to monitor clonal dynamics within *Plasmodium* infections become more widely available, windows will be opened into our understanding of the complex ecology of malaria parasites.

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