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Clonal diversity alters the infection dynamics of a malaria parasite (*Plasmodium mexicanum*) in its vertebrate host

Anne M. Vardo-Zalik[1] and Jos. J. Schall

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

Abstract. Ecological and evolutionary theory predicts that genetic diversity of microparasites within infected hosts will influence the parasite replication rate, parasitemia, transmission strategy, and virulence. We manipulated clonal diversity (number of genotypes) of the malaria parasite, *Plasmodium mexicanum*, in its natural lizard host and measured important features of the infection dynamics, the first such study for any natural *Plasmodium*–host association. Hosts harboring either a single *P. mexicanum* clone or various combinations of clones (scored via three microsatellite markers) were established. Production of asexually replicating stages (meronts) and maximal meront parasitemia did not differ by clonal diversity, nor did timing of first production of transmission stages (gametocytes). However, mean rate of gametocyte increase and maximal gametocyte parasitemia were greater for hosts with mixed-clone infections. Characteristics of infections were more variable in hosts with mixed-clone infections than with single-clone infections except for first production of gametocytes. One or more of the parasite reproductive traits were extreme in 20 of 52 hosts with mixed-clone infections. This was not associated with specific clones, but diversity itself. The overall pattern from studies of clonal diversity for human, rodent, and now reptile malaria parasites confirms that the genetic diversity of infections in the vertebrate host is of central importance for the ecology of *Plasmodium*.

Key words: clonal diversity; infection dynamics; malaria parasite; Plasmodium; Plasmodium mexicanum.

Introduction

Microparasites (viruses, bacteria, and protists) replicate within an individual host as either a single genetically uniform clone, or as a mixture of clones (Anderson and May 1979, Read and Taylor 2001). Many theoretical studies, both verbal and mathematical, reason that clonal diversity within infected hosts will play a central role in the ecology of microparasites and their hosts (May and Nowak 1995, Van Baalen and Sabelis 1995, Frank 1996, Read and Taylor 2000, 2001, Schall 2002). One common view is that high clonal diversity will be associated with rapid parasite replication and greater final parasite loads, leading to an increase in pathology suffered by the host (Bull 1995, May and Nowak 1995). If this is true, the dynamics of infection associated with clonal diversity may influence the parasite's transmission success and prevalence in the host, with cascading effects on host populations (Taylor et al. 1997, Eisen and Schall 2000, Schall 2000). Despite the possible importance of clonal diversity for the biology of parasites, including important pathogens of humans and wildlife species, data relating the number of genotypes in an infected host to the parasite's infection dynamics are surprisingly scant (Read and Taylor 2001). We conducted an experimental study manipulating the number of coexisting clones of the lizard malaria parasite, *Plasmodium mexicanum*, in its natural vertebrate host, the western fence lizard, *Sceloporus occidentalis* (Schall 1996). Our goal was to evaluate several competing views on how clonal diversity may influence the dynamics of microparasite infections.

Malaria parasites (*Plasmodium*) cycle between two hosts, a vertebrate (squamate reptile, bird, or mammal), and an insect vector (mosquito or sandfly) (Valkiunas 2005). Within vertebrate hosts, malaria parasites undergo bouts of asexual replication of haploid cells (meronts), and the proportion of blood cells infected (parasitemia) increases over time to a maximal level, which may hold constant or decline as the immune system becomes active. A fraction of the parasites develop into gametocytes, the nonreproducing cells transmitted to biting insect vectors in blood meals (Eisen and Schall 2000). The replication rate of the asexual parasite cells, timing of the shift to production of gametocytes, and greatest meront and gametocyte parasitemia of every *Plasmodium* species studied to date vary substantially among naturally infected hosts (Eisen and Schall 2000). For example, maximal parasitemia of *P. mexicanum* infections varies from <1% to 90% of erythrocytes infected (Bromwich and Schall 1986, Eisen

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[1] Present address: Program in Public Health, Room 3501, Hewitt Hall, University of California, Irvine, California 92697 USA. E-mail: avardoza@uci.edu

2000). Clonal diversity also varies among *Plasmodium* infections. Many studies on human malaria parasites (e.g., Anderson et al. 2000, Imwong et al. 2006, Bruce et al. 2007), and rare data on the *Plasmodium* of nonhuman vertebrates (*P. mexicanum*; Vardo and Schall 2007), document that infected vertebrates may harbor one to many parasite clones. For *P. mexicanum*, mixed-clonal infections were found in 50% to 88% of infected lizards (Vardo and Schall 2007). Thus, malaria parasites provide excellent models to study the relationship between the dynamics of infection and clonal diversity.

Clonal diversity could influence the course of *Plasmodium* infections in several ways. Single-clone infections could proceed by balancing between loss of infected blood cells removed from circulation and production of gametocytes for transmission. That is, the course of infection would follow some optimal path to insure maximal possible transmission (Eisen and Schall 2000, Schall 2002). But in mixed-clone infections, several outcomes are possible. First, competition for limiting resources within the host could limit overall parasite growth rate and maximal parasitemia for both single- and mixed-clone infections. Each clone would therefore suffer reduction in density caused by competition, but the overall parasitemia would be similar for single-clone and mixed-clone infections (de Roode et al. 2003). Second, mixed-clone infections could be more productive, with higher replication rates and higher maximal parasitemia, because the final parasitemia is a sum over all clones (no interaction) (Schall 2000, de Roode et al. 2003), because clones increase replication rates (competition for transmission), or because they more readily elude the immune system (Taylor et al. 1998, Eisen and Schall 2000, Ferguson et al. 2003). Third, competition among clones may be severe and reduce the final overall parasitemia, especially if the mixture of parasite genotypes elicits a stronger immune response by the host (de Roode et al. 2005). Last, superimposed on all these possible outcomes, competition among clones for transmission to the vector could lead to earlier production of gametocytes in mixed-clone infections.

Evidence for the role of clonal diversity on infection dynamics of malaria parasites emerges from surveys of natural human malaria infections and experimental studies of laboratory model systems. Studies of human malaria cannot experimentally alter the clonal diversity within infected hosts, and by necessity only glimpse events within each host (Ferreira et al. 2002). A more controlled picture comes from manipulative experiments using laboratory model systems, such as the African rodent malaria parasite *P. chabaudi* in laboratory mice (Mackinnon and Read 2004). Laboratory model systems, however, do not use natural parasite–host associations that have experienced coevolution to face challenges experienced in a natural setting. We examined the influence of clonal diversity on *P. mexicanum* by determining the replication rate of meronts, maximum meront parasitemia, timing of first production of gametocytes, rate of gametocyte increase, and maximum gametocyte parasitemia within hosts carrying infections of *P. mexicanum* (Bromwich and Schall 1986, Eisen 2000).

Materials and Methods

Study site and collection of lizards

The study was conducted at the University of California Hopland Research and Extension Center (hereafter Hopland), a 2160-ha tract of oak woodland in southern Mendocino County, California, USA. Hopland has been the focus of a long-term study from 1978 to 2007 on the biology of *P. mexicanum* in its hosts, the western fence lizard, *Sceloporus occidentalis*, and two psychodid sandflies, *Luzomyia vexator* and *L. stewardii* (Fialho and Schall 1995, Schall 1996, 2002).

During May, 2005, experimental infections were initiated in 69 noninfected adult male fence lizards (snout-to-vent length 65–75 mm) collected from two sites at Hopland where *P. mexicanum* was absent in the lizards since 1978 (39°02′05″ N, 123°05′49″ W and 38°59′27″ N, 123°05′27″ W) (Schall and Marghoob 1995; J. J. Schall, *unpublished data*). To insure that recipient lizards were not infected, blood smears (stained with Giemsa) were scanned at 1000× to examine >10 000 erythrocytes. Also, a PCR-based protocol that detects infections that are not detectable by microscopy (as low as one parasite cell per millions of erythrocytes) confirmed the noninfected status of these lizards (Perkins et al. 1998, Vardo et al. 2005). A control group of 19 noninfected male lizards from the collecting sites was kept for the study's duration, and all remained noninfected by both microscopy and PCR analysis. Infected donor lizards were identified within a sample of 484 lizards collected from sites where malaria has been most common over the past three decades (all within 1.2 km of 39°00′02″ N, 123°04′39″ W). Infected lizards with high parasitemia of replicating meronts (>25 parasites/1000 erythrocytes) were rare during the sample period because prevalence was low in 2005 (~6%) and such infections are always uncommon early in the warm season (Bromwich and Schall 1986). Only five lizards were suitable to serve as donors of infected blood. Two measures of lizard health were taken for each animal prior to initiation of experiments: proportion of immature erythrocytes (Schall 2002) and tail condition (normal, broken, regenerated).

Genotyping infections

Blood samples from the donor and experimentally infected lizards were taken from a toe clip and stored frozen as dried dots made on filter paper (Vardo and Schall 2007). DNA was extracted from dried dots with the Qiagen DNeasy kit (Qiagen, Valencia, California, USA). Three microsatellite markers were amplified (*Pmx306*, *Pmx732*, and *Pmx747*) using primers and PCR conditions in Schall and Vardo (2007), and Ready-to-Go PCR beads (GE Health Care, Piscataway, New

TABLE 1. Number of clones in experimentally infected lizards and the donor lizards used to induce replicate infections of *Plasmodium mexicanum*.

Treatment	Donor(s)	*N*
1A	I	7
1B	II	7
1C	III	3
2	I+II	8
2–4	III	5
2–8	IV	7
4–32A	III–IV	8
4–32B	III+IV+V	14
6–60	I+II+III+IV	10

Notes: Nine treatment groups are listed, based on number of clones present (three single-clone treatments, with different genotype of parasite present, and two 4–32 clone treatments with different assemblages of clones). Donor infection (Table 2) is given, and the number of clones present is presented as a range (minimum–maximum number of clones). Treatment group 1C received blood from an infected host containing 2–4 clones, but only a single clone became established. Sample size (*N*) of replicate infections (lizards) for each treatment is listed.

Jersey, USA). Samples were processed on an ABI 310 genetic analyzer, and data analyzed with GeneMapper 3.5 software (ABI, Foster City, California, USA). Because the parasite stages in the vertebrate host are haploid, each peak on the pherogram represents a single parasite clone (Anderson et al. 2000, Vardo and Schall 2007). A sample with a single allele at each marker was scored as a "single clone" infection. Previous experience (Vardo and Schall 2007) indicated that increasing the number of markers to six did not detect additional clones. A sample with two alleles at a single marker and one at the other two markers was scored as a "two clone" infection. Samples with two or more alleles at >1 marker were problematic. To score clonal diversity in these, we calculated the minimum and maximum number of clones possible, based on the three markers. The minimum number of clones within a sample is the maximum number of alleles seen for any of the markers (Anderson et al. 2000, Vardo and Schall 2007). The maximum present is the product of the number of alleles seen across the three markers. We report number of clones as the range of possibilities. Blood samples taken at 30 days and 80 days postinjection for each recipient lizard were processed to score the number of clones that had become established. Three of the lizards lost a clone after they received two clones, so they were moved to a "one clone" treatment group (Table 1).

Experimental infections

Lizards with single-clone infections are uncommon at the site (~15% of infected lizards; Vardo and Schall 2007), and lizards used to provide infected blood are small (~10 g). Therefore, large (and equal) sample sizes were not possible for all treatment groups. Five donor lizards were selected, two with a single clone (Donors I and II, each with a different genotype), one with two clones (Donor III with two genotypes not present in Donors I and II), and two with 2–8 possible clones (Donors IV and V), each harboring at least one unique genotype (Table 2). Thus, within all five donors, 7–15 clones were available for study (Table 2).

Experimental infections were initiated via intraperitoneal injection of donor blood (Osgood and Schall 2003). Briefly, blood taken from a donor lizard was mixed with phosphate-buffered saline, and the sample diluted to contain 2×10^5 meronts in 20 mL of blood/saline mix. Blood from multiple donors was mixed to insure that the same number of parasite cells from each donor was injected into recipient lizards.

The nine treatment groups, with information on specific alleles present and sample sizes, are given in Table 1, and are designated by the number of clones. The recipient lizards and control lizards were housed in 12 large outdoor vector-proof cages, and fed each day to satiation with mealworms and crickets. Each treatment group was housed in separate cages, randomly moved each week in the 5×2 m array.

Infection traits

Blood samples, obtained from a toe clip, were taken every 10 days over a three-month period from June to August (80 days from injection of blood into the recipients). To reduce major physiological cost to the lizards, and possible alterations in the course of *P. mexicanum* infection, only two small drops of blood were drawn, one to make a blood smear and one on filter paper to be stored dried and frozen.

The course of a *P. mexicanum* infection typically reaches maximal parasitemia and maximal production of gametocytes in 60–90 days, and thus samples taken every 10 days reveal events during the course of infection (Eisen and Schall 2000, Osgood and Schall 2003). Slides were examined to count the number of parasites seen in 1000 erythrocytes from all areas of the smear, scoring number of meronts and gametocytes. Infections followed two patterns: parasitemia rose to a peak, then dropped (often a stable lower point), or rose to a high stable level. Data for each infection were examined to score the dates on which the infection reached its highest

TABLE 2. Genotypes of the malaria parasite *Plasmodium mexicanum* within donor lizards, *Sceloporus occidentalis*, used to initiate replicate experimental infections.

Donor	*Pmx306*	*Pmx732*	*Pmx747*	*N* clones
I	A	5	c	1
II	E	5	a	1
III	C, F	2, 4	c	2–4
IV	B, D	6, 1	b, c	2–8
V	C	2, 1	c	2

Notes: Three microsatellite markers were genotyped to score the number of clones present. Alleles for each locus are labeled from smallest (shortest repeat) to longest (A–D for locus *Pmx306*, 1–6 for locus *Pmx732*, and a–c for locus *Pmx747*; loci are described in Schall and Vardo [2007]). Number of clones present (*N* clones) is reported as the minimum to maximum that could be present (see *Materials and Methods*).

TABLE 3. Traits for *Plasmodium mexicanum* in hosts infected with a different numbers of parasite genotypes (clones).

Treatment group	Maximum meront parasitemia	Maximum gametocyte parasitemia	Meront rate of increase	Gametocyte rate of increase	Total meronts produced over time	Total gametocytes produced over time
1A ($N = 7$)	31.4 (20.1), 10–68	21.0 (11.3), 8–39	0.92 (0.59), 0.20–1. 90	0.69 (0. 47), 0.15–1.50	83 (51.4), 30–179	66.9 (52.2) 21–175
1B ($N = 7$)	26.0 (20.7), 8–66	7.6 (6.5), 1–18	0.68 (0.58), 0.13–1.60	0.40 (0.29), 0.03–0.75	62.7 (51.6), 27–174	20.6 (22.2) 2–57
1C ($N = 3$)	15.3 (3.2), 13–19	13 (10.1), 4–24	0.58 (0.11), 0.45–0.65	0.66 (0.47), 0.37–1.20	38.3 (10.7), 29–50	33.3 (21.2) 11–69
2 ($N = 8$)	102.1 (139.8), 5–350	65.1 (95.2), 4–278	2.4 (2.25), 0.28–5.74	2.37 (2.65), 0.20–6.95	157.0 (257.3), 13–737	103.6 (113.1) 12–320
2–4 ($N = 5$)	64.2 (70.1), 7–179	23.2 (13.5), 9–42	1.09 (1.10), 0.11–2.93	0.79 (0.37), 0.45–1.40	170.8 (148.8), 25–340	67.8 (39.3) 27–117
2–8 ($N = 7$)	92.0 (134.4), 4–345	35.3 (32.3), 3–78	1.80 (2.39), 0.10–6.76	1.67 (1.31), 0.35–3.80	120.7 (201.2), 8–525	80.8 (76.8) 7–189
4–32A ($N = 8$)	88.1 (73.3), 14–235	34.0 (27.7), 13–91	1.54 (1.13), 0.35–3.85	1.05 (0.92), 0.21–3.03	171.1 (91.3), 37–271	70.7 (45.7) 28–152
4–32B ($N = 14$)	45.4 (48.9), 7–139	22.2 (19.7), 5–77	1.11 (1.02), 0.16–3.48	0.99 (0.92), 0.17–3.80	105.9 (110.9), 21–369	98.6 (147.1) 12–578
6–60 ($N = 10$)	27.9 (32.9), 4–111	46.3 (63.1), 5–207	0.75 (0.86), 0.10–2.48	2.12 (3.17), 0.23–10.40	95.0 (128.8), 10–436	132.6 (176.0) 19–574
1 ($N = 17$)	26.2 (18.4), 8–68	14.1 (10.8), 1–39	0.76 (0.53), 0.13–1.90	0.56 (0.41), 0.03–1.50	66.8 (47.7), 27–179	41.9 (42.6) 2–175
>1 ($N = 52$)	65.4 (86.6), 4–350	37.1 (50.1), 3–278	1.39 (1.52), 0.10–6.76	1.50 (1.90), 0.17–10.40	128.7 (150.9), 8–737	96.9 (121.5) 7–578

Notes: Treatment groups are labeled as in Tables 1 and 2. In the final two rows, results are presented as summaries of all single-clone or multiple-clone treatments. Means (SD) and ranges are $\log_{10}$ back-transformed after analysis and samples sizes for each treatment are given (N).

meront and gametocyte parasitemia. For infections that maintained high constant parasitemia, the first date was chosen to score peak parasitemia. However, infections that reached this peak, then dropped, would produce fewer parasites over time than those that maintained their highest parasitemia for weeks. Therefore, for lizards that lived the full 80 days of the experiment (66 of 69), total parasites seen in 1000 erythrocytes were summed for all samples as a second measure of maximal parasite production. The rate of increase of both meront and gametocyte parasitemia was calculated as the highest parasitemia (first date for those reaching a stable point) minus the parasitemia when parasites were first observed on the smears divided by the number of days between these two samples. First production of gametocytes was scored as the days from injection of infected blood into the lizard to appearance of gametocytes, and the days from first appearance of meronts in the blood to the first appearance of gametocytes.

Analysis

All analyses of infection traits were performed by nested ANOVA. This method partitioned variance by treatment group (number of clones in the infected lizard) and replicate within treatment group (different donor or sets of donors). A significant replicate effect would indicate some effect of specific clones rather than number of clones. Unequal sample sizes in a nested ANOVA increase the likelihood of a Type II error (Sokal and Rohlf 1969), which may be reduced by considering the replicates as random effects and using a restricted maximum likelihood (REML) approach and the Satterthwaite method to estimate degrees of freedom (Dudley et al. 2007). However, this does not allow examination of the replicate effect (the specific clones involved). Therefore, we performed both an uncorrected nested ANOVA and the REML analysis. Qualitative results were similar, and we report here only the uncorrected ANOVA. Five main treatments were 1 clone (three trials), 2 clones (one trial), 2–8 clones (two trials: 2–4 and 2–8 clones), 4–32 clones (two trials), and 6–60 clones (one trial). Data were nested as individual infected hosts, within replicate trials, within main treatment groups. Infection traits were entered into a principal component analysis to combine possible correlation among traits. Data for all infection traits were not normally distributed, and so for ANOVA they were $\log_{10}$-transformed. Transformed data were not significantly different from normal in distribution (Shapiro-Wilk goodness-of-fit test, Bartlett test, $P > 0.05$). Some data could not be rendered normal by any transformation (first production of gametocytes, site of capture, proportion immature red blood cells, tail status, and principal component scores), and thus nonparametric Kruskal-Wallis and Mann-Whitney tests were performed. All analyses were conducted using the JMP statistical package.

RESULTS

Growth rates, maximal parasitemia, and first production of gametocytes

Data for the treatment groups are given in Table 3 and Fig. 1 and results of ANOVA in Table 4. Meront

growth rate, maximal meront parasitemia, and sum of meront parasitemia over all sample periods did not differ between main treatment or replicate trials nested within the main treatment groups. Grouping data as single vs. mixed-clone infections also revealed no difference in meront growth rate and maximal parasitemia. Similarly, rate of increase of gametocytes, maximal gametocyte parasitemia, and sum of gametocytes over all samples did not differ across main treatment groups. However, comparison between hosts with single-clone infections ($N = 17$ hosts) to all hosts with mixed-clone infections ($N = 52$) revealed greater gametocyte growth rate and maximal gametocyte parasitemia in hosts with mixed-clone infections.

Rate of increase of *P. mexicanum* meronts and gametocytes and their maximal parasitemia were previously found to be correlated (Eisen and Schall 2000), and this was confirmed in these experimental infections (correlations, $r = 0.51$ [maximal meronts vs. rate of gametocyte increase] to 0.74 [gametocyte increase vs. maximal gametocyte parasitemia]). Therefore, the traits were combined in principal components analysis. A single factor that included the four rates and maximal parasitemia traits accounted for 74% of overall variance, very similar to the result of Eisen and Schall (2000). Analyses comparing treatments by the principal component score mirrored those for single traits: among all treatment groups no differences were found between medians of treatment groups (Kruskal-Wallis test, $P = 0.25$), but traits from single-clone and all mixed-clone infections differed (Mann-Whitney test, $P = 0.049$).

Timing for first appearance of gametocytes varied from 10 to 70 days after injection across all experimental infections, and from 0 to 40 days after the first appearance of any parasites in the blood (a few infections produced gametocytes for the first time when meronts first appeared in the blood). However, clone number did not influence the timing of first gametocytes (Kruskal-Wallis tests for both measures of first gametocytes, $P > 0.05$). For example, medians for days between injection of blood and first gametocytes were 40 for six of nine treatments, with a range of 35–70.

Variation in traits

Examination of the raw, untransformed data revealed a difference in variance for meront and gametocyte rates of increase and for their maximal parasitemias between hosts with single-clone and mixed-clone infections (Bartlett tests, all $P < 0.001$; Fig. 1). For the infection traits shown in Fig. 1, we defined "high" infections as those with values $> x + \text{SD}$ for the total data set of 69 infections, and "low" infections as those values $< x + \text{SD}$. No hosts with the single-clone infections fell in the high group, but 20 of the 52 mixed-clone hosts (39%) were high for one or more of the infection traits. We asked if hosts with the high infections for one trait were also the high infections for other traits. Only four fell into the high class for all traits, whereas nine infections

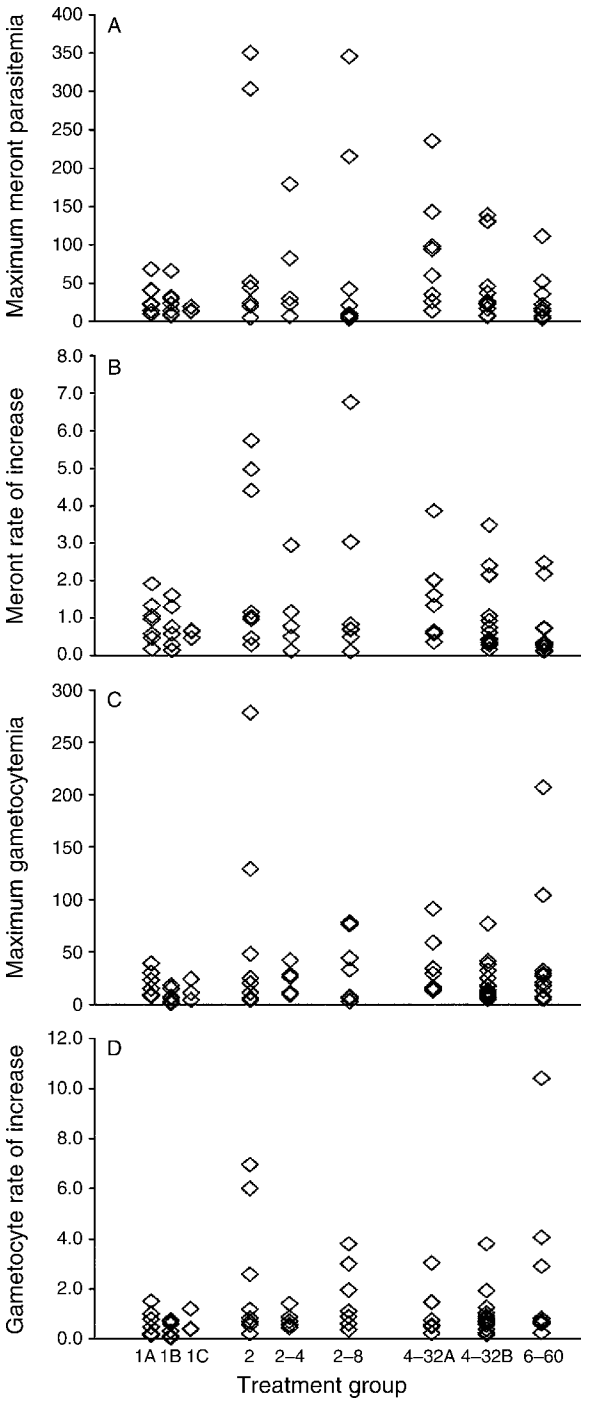

FIG. 1. Four infection traits of the malaria parasite, *Plasmodium mexicanum*, within the vertebrate host (fence lizards) when experimental infections contain one or multiple genetic clones of haploid parasite cells. The number of clones in experimentally infected hosts was to 6–60 clones, and replicate treatment groups are indicated by letters (Table 1). (A) Maximal density (parasitemia) of asexually replicating *P. mexicanum* cells (meronts) during an 80-day period (parasites/1000 erythrocytes). (B) Rate of increase of meronts (parasites·d^{-1}·1000 erythrocytes^{-1}). (C) Maximal parasitemia of transmission stages (gametocytes/1000 erythrocytes). (D) Rate of increase of gametocytes (cells·d^{-1}·1000 erythrocytes^{-1}).

TABLE 4. ANOVA of main treatments (number of clones per host) and nested replicate treatments within the main treatment groups for several infection traits of *Plasmodium mexicanum* in its natural vertebrate host.

	All treatment groups						1 vs. >1 clone					
	Main treatment			Replicate group			Main treatment			Replicate group		
	F	df	*P*	*F*	df	*P*	*F*	df	*P*	*F*	df	*P*
Rate of increase of meront stages	1.24	8, 60	0.10	1.24	8, 60	0.74	1.47	8, 60	0.23	1.20	8, 60	0.31
Maximum meront parasitemia	1.18	8, 60	0.12	1.18	8, 60	0.51	2.31	8, 60	0.13	1.13	8, 60	0.36
Sum of meronts over all samples	1.09	8, 57	0.33	1.09	8, 57	0.24	1.62	8, 57	0.21	1.12	8, 57	0.36
Rate of increase of gametocytes	1.59	8, 60	0.09	1.59	8, 60	0.67	6.17	8, 60	0.02	0.64	8, 60	0.72
Maximum gametocyte parasitemia	1.91	8, 60	0.11	1.91	8, 60	0.17	6.99	8, 60	0.01	1.07	8, 60	0.39
Sum of gametocytes over all samples	2.06	8, 57	0.10	2.06	8, 57	0.12	6.72	8, 57	0.01	1.21	8, 57	0.31

were high for only one trait, three for two traits and four for three traits. Thus, high infections were not the same infections for all four reproductive traits of infections. Clonal diversity may have no role in the 20/69 (29%) total infections being high for at least one infection trait; that is, by chance the 17 single-clone infections may have been drawn from the 71% of overall infections that had lower productivity. This has a low likelihood (the probability of randomly drawing a sample of 17 low infections from a population that contains 71% low infections $= 0.71^{17} = 0.003$).

It is possible that specific alleles at one or more markers are associated with higher growth rates or higher parasitemia, and hosts with mixed-clone infections could be more likely to contain these genotypes by chance. Ideally, each clone within a mixed-clone infection would also be tested when it is the only infecting genotype, but this was not possible because of the small number of suitable donor infections available in the study. We therefore examined the data in several ways to detect any hint of a clone effect. First, the ideal situation was available for treatment group 2, which contained the two clones found in two of the single-clone groups (groups 1A and 1B). Treatment group 2 included infections that were high in three of four life history traits, but the donor infections (1A and 1B) did not produce exceptionally high rates of increase or maximal parasitemia. Second, for all three microsatellite markers, most alleles were found in lizards with both the high and low infections. For example, for asexual growth rate, six of six *Pmx306* alleles were seen in hosts with both the high and low infections, four of six alleles for *Pmx732* were seen in both groups, and four of five alleles for *Pmx747*. Similar results were seen for the other traits. Last, the same multi-allele combinations for each marker were found in both the high and low infections. For meront growth rate, six pairs of hosts (one with high and one with low) had the same combination of alleles, for gametocyte growth rate, seven pairs had the same combination, for maximum asexual parasites, five pairs matched, and for maximum gametocytes, five pairs matched. Thus, no hint is seen for any allele-specific effect on the infection traits.

Other factors, not related to the infection clonal diversity, also could have influenced infections to fall into the high group, such as a host variation effect. Lizard size (a measure of age) did not differ between high and low groups (U test, $P = 0.63$), nor did site of capture (lizards from the same local area) (χ^2 test, $P = 0.27$). Two measures of health were also not associated with the infection being high or low: proportion of erythrocytes immature (U test, $P = 0.73$), and tail condition (χ^2 test, $P > 0.05$).

DISCUSSION

Several outcomes are possible when multiple clones of *Plasmodium* parasites co-occur within infected hosts. (1) The rate of increase and maximal parasitemia for both meronts and gametocytes could be the same for mixed- and single-clone infections because resources limit overall parasite density. (2) Mixed-clone infections could be more productive than single-clone infections because each clone simply reaches its optimal density (and clones sum), because each clone increases its rate of replication to maximize its chance of being transmitted, or because all clones experience a weaker immune attack. (3) All clones suffer severe competition when they coexist in a host, and thus mixed-clone infections would be less productive, with lower rates of increase and maximal parasitemia. Superimposed on these possibilities would be shifts in the first production of gametocytes as a mechanism of competition for transmission into the vector. We sought to determine which of these possible outcomes obtain when the clonal diversity of *P. mexicanum* infections was experimentally altered.

Infection traits of *P. mexicanum* did not differ across the five main treatments (number of clones). Combining all mixed-clone infection treatments, no differences were observed in average increase or maximal parasitemia of asexually replicating meronts, but gametocytes increased more rapidly and reached greater parasitemia in the mixed-clone infections. This means that meront replication rate must also increase in the mixed-clone infections to convert a greater proportion to gametocytes, and that the effect is similar for two to many clones. The timing of the first occurrence of gametocytes in the blood did not change as clonal diversity increased. Even more striking, the rate of increase and maximal parasitemia of both meronts and gametocytes was more variable in the mixed-clone infections. All of the single-clone infections

fell within one standard deviation for these traits, but 39% of mixed clone infections were extreme (>1 SD from overall mean) for at least one infection trait.

The results allow evaluation of the possible effects of clonal diversity on the course of infection predicted by the verbal theory. Final parasitemia of both gametocytes and meronts is often higher in mixed-clone infections, indicating that interference among clones does not limit final parasite density to the same or lower levels than seen in single-clone infections. Also, the parasitemia in mixed-clone infections is often greater than expected from a sum of all clones. For example, maximal gametocyte parasitemia for infections with two clones is more than twice that from simply summing the maximum from two individual clones (Table 3). The results suggest that clones of *P. mexicanum* often increase their rate of replication and final abundance when they coexist with other genotypes of parasite. This could be a product of either some competitive race to favor each clone's transmission success or the benefit of a reduced immune attack for complex infections.

Evidence for competition among *Plasmodium* clones is seen in studies of human, rodent, and now reptile malaria parasites, although the outcome of competition varies enormously. For people suffering chronic infection of *P. falciparum*, sudden onset of serious illness follows arrival of a novel genotype, with no indication that specific genotypes are more virulent (Roper et al. 1998, Ferriera et al. 2002, Bruce et al. 2007). Pathogenesis does not depend on high clonal diversity, but is simply triggered by the arrival of a new clone. Indeed, genetically diverse infections appear more successful in restricting entry of novel clones (Smith et al. 1999). Competition among clones of the rodent malaria parasite, *P. chabaudi*, is intense in a laboratory mouse host, but the outcome depends on specific genotypes present and their order of entry into the host (e.g., de Roode et al. 2003, 2005). A manipulative experiment demonstrated between-clone competition in *P. mexicanum*; novel genotypes were excluded from entry into already infected hosts (Vardo et al. 2007). The experiment presented here shows that once multiple clones enter a host and become established, rate of replication and final parasitemia of each clone will change.

The tidy conclusion that high clonal diversity of *P. mexicanum* within hosts leads to greater parasite replication and parasitemia is confounded by the variation observed in the mixed-clonal infections. Only some mixed-clone infections were highly productive, whereas other infections followed a course typical of single-clone infections. If either competition for transmission success or cooperation to elude the immune system results in higher replication rates and parasitemia, why do not all mixed-clone infections respond? Arrival of a novel clone in some infections (with results similar to that seen for *P. falciparum* infections in humans; Smith et al. 1999), is highly unlikely because the experimental lizards were housed in vector-proof cages, so new clones could not have been transmitted to the lizards. Also, no new genotypes appeared in hosts based on samples taken at 30 and 80 days after inoculation. Alternatively, a host effect is possible if lizards collected from the wild population varied in genotype or immune competency. Experiments comparing infections of *P. chabaudi* in different mouse strains or those with different immune competence reveal variation in the course of infection among treatment groups (Mackinnon and Read 2003, de Roode et al. 2004).

The timing of first production of gametocytes was uniform across all the experimental treatments. This may be unexpected because stress leading to increases in mortality of younger age classes should lead to shifts toward early reproduction (Roff 1992). Remarkably, *P. chabaudi* shifts to early production of gametocytes when challenged with antimalarial drugs (Buckling et al. 1997). Clonal diversity itself does not appear to cause a similar shift in *P. mexicanum*, at least in the time scale of these experiments. Instead, mixed-clone infections of *P. mexicanum* produce gametocytes at a more rapid rate and achieve higher gametocyte densities.

Read and Taylor (2001) found few empirical tests of the view that genetic diversity within microparasite infections should alter the dynamics of infection including rate of replication, parasite density, transmission strategy, and virulence. Studies of natural *Plasmodium* infections in humans, experiments using a laboratory model of rodent *Plasmodium* in mice, and now the first manipulative study of clonal diversity for a natural parasite–host association all agree that the number of genotypes of the parasites within the vertebrate host plays an important role in the course of those infections.

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