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ESTABLISHMENT EFFICIENCY AMONG CLONES OF THE MALARIA PARASITE, *PLASMODIUM MEXICANUM*, FOR MIXED-CLONE INFECTIONS IN ITS NATURAL LIZARD HOST

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ABSTRACT: Within genetically diverse infections of malaria parasites (*Plasmodium* spp.), the relative proportions of genetic clones in the vertebrate host's blood can influence clonal competition, transmission success, gametocyte sex ratio, and virulence. Clonal proportions depend on establishment success of each clone when they enter a new host and on subsequent differences in rates of asexual replication and clearance. Both of these life history traits could be influenced by clone genotype. To assess genetic (clonal) influences on both establishment success and later changes in relative proportion for the lizard malaria parasite *Plasmodium mexicanum*, 7 naturally infected fence lizards harboring a single clone of *P. mexicanum* served as donors to initiate replicate experimental infections containing each of the clones and combinations of 2 clones. Measured were relative establishment success of each clone, change in relative proportions over time, and rate of increase of parasite density and total parasitemia. Relative clonal proportions were determined using microsatellite markers. Rates of increase in the parasitemia and degree of change in relative proportions were not correlated, so both rapidly and slowly growing infections could show either little or substantial change in clonal proportions over time. There was a significant clone effect on establishment efficiency but not on later changes in relative proportions. These results argue for a combination of genetic and environmental (host) effects on the success of *P. mexicanum* clones in genetically complex infections. The maintenance of genetic variation for establishment success, but not subsequent replication rate or shifts in relative proportion, suggests trade-offs between these traits during life history evolution of malaria parasites.

When multiple, genetically-distinct clones of a microparasite, such as the malaria parasites (*Plasmodium* and related genera, sensu Martinsen et al., 2008), coexist within a single host they may interact in complex ways, some interactions being antagonistic while others could be cooperative (Read and Taylor, 2001). Molecular techniques now reveal that mixed-clone infections of malaria parasites are common in individual vertebrate hosts (Anderson et al., 2000; Conway, 2007; Vardo and Schall, 2007; Havryliuk and Ferreira, 2009; Koepfli et al., 2011). From the earliest days of the study of *Plasmodium*, researchers suspected that interaction among coexisting clones would alter the dynamics of infections (Wenyon, 1926), and now many studies support that idea, revealing influences on virulence (Taylor et al., 1998; Branch et al., 2001; Vardo-Zalik and Schall, 2008), replication rate (Vardo-Zalik and Schall, 2009), and drug resistance (Huijben et al., 2010). In addition to the number of clones present, the relative proportions of those clones is also important for the ecology of malaria parasites, such as competitive interactions (de Roode et al., 2005a, 2005b; Bell et al., 2006), transmission success into the vector (Taylor et al., 1997; Wargo et al., 2007), and gametocyte sex ratio (Reece et al., 2008; Schall, 2009). How coexisting clones reach their relative proportions appears to be shaped by complex, but poorly understood, processes including establishment efficiency when clones enter a new host and the replication rates and clearance rates of parasite cells (combined to yield the clonal rate of change). These in turn would be influenced by the parasite's genetic variation, variation in host environment, and strictly stochastic events. Particularly interesting is how clonal proportions may shift over time because this would offer insight into how clones interact among themselves and with the host immune system.

Most studies on clonal diversity of malaria parasites are limited to determining simply the number of clones present in an infected host's blood but not their relative proportions. Determining clonal proportions for human malaria parasites has proven vexing. Proportions of clones in the blood change as the parasites sequester and then reappear in the blood (*Plasmodium falciparum*; Farnert, 2008) or when genotypes of parasites emerge from the liver to appear in the blood for the first time (*Plasmodium vivax*; Chen et al., 2007). This confounds not only efforts to determine relative proportions of clones but even how to define relative proportions. These problems may not apply for the other human malaria parasites, although to our knowledge clonal proportions have not been measured for those species. A clearer picture emerges from using reverse-transcription and real-time PCR for a rodent malaria parasite in laboratory mice. Using these methods, relative proportions of well-characterized genotypes of *Plasmodium chabaudi* have been quantified in studies on clonal competition and changes in gametocyte sex ratios (Cheesman et al., 2003; Drew and Reece, 2007; Reece et al., 2008).

We focus here on the relative abundance of coexisting clones of a lizard malaria parasite, *P. mexicanum*, which naturally infects western fence lizards (*Sceloporus occidentalis*) in California, United States. Previous studies revealed several intriguing patterns in the life history of *P. mexicanum*. First, a mark–recapture program showed the parasite growth rate varies greatly among lizard hosts, and final equilibrium parasitemia varies over 3 orders of magnitude (Bromwich and Schall, 1986). Second, mixed-clone experimental infections tend to have higher growth rates and maximum parasitemia, but there does not appear to be any clone-specific effect on these traits (Vardo-Zalik and Schall, 2009). Third, relative clonal proportions change little in about 2/3 of infections once the infection becomes established and, when proportions do change, the change is typically slow (Ford and Schall, 2011). Infections with only slight changes in proportions are most often observed when only 2 genotypes are present and are seen in infections with both rapidly and slowly growing parasitemia (Ford et al., 2010; Ford and Schall, 2011). For infections with changing relative proportions, in most cases the less-common clone increases over time, suggesting those clones experience more-rapid growth rates or are less prone to immune

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clearance. These patterns, though, argue that relative success of each clone depends more often on establishment ability rather than on differential growth.

We initiated replicate 2-clone infections with combinations of clones that were isolated from single-clone natural infections and followed the relative proportions of clones over the course of the infection. We asked if there is a clone effect on establishment success of *P. mexicanum* or any change in relative proportions of clones (or both). In addition we asked if change in relative clonal proportion is indeed independent of rate of increase in parasite density in the lizard blood as reported previously (Ford and Schall, 2011). Our goal was to determine if establishment efficiency, an important life history trait, retains genetic variation in the parasite population despite likely strong selection favoring the more efficient genotypes.

MATERIALS AND METHODS

The study was conducted at the University of California Hopland Research and Extension Center (HREC) in Mendocino County, California, where *P. mexicanum* has been under study in its lizard host, the western fence lizard *S. occidentalis*, since 1978 (Bromwich and Schall, 1986; Schall, 1996; Fricke et al., 2010; Schall and St. Denis, 2013). Not-infected lizards, which served as hosts for the experimental infections, were collected from sites with historically very low prevalence of infection (Schall and Marghoob, 1995). Two methods were used to ensure that recipient lizards were not harboring infections prior to the experiment. First, a toe clip provided blood for a thin blood smear which was treated with Giemsa stain and scanned at ×1,000 to examine >5,000 erythrocytes for parasites. If a lizard was scored as not infected, a second test used PCR of DNA extracted from blood to amplify a segment of the *Plasmodium* cytochrome *b* gene, and the product was run on a gel to look for presence of the expected-sized amplicon. This method can detect infections at very low parasitemia (Vardo et al., 2005). All recipient lizards were males and ~60 mm snout–vent length, and were thus animals that had hatched the previous season (based on mark–recapture data).

Infected lizards were collected from sites where parasite prevalence has been highest in the area over the past 3 decades (Schall and Marghoob, 1995; Vardo and Schall, 2007) and were selected after examination of stained blood smears. To initiate the experimental infections, donor lizards were identified based on presence of a single genetic clone of parasites and high parasitemia of asexual stages (>15 asexual stages/1,000 erythrocytes on a blood smear). Seven lizards met these criteria and were infected with only a single clone of *P. mexicanum* identified by analysis of 4 highly polymorphic microsatellite loci, Pmx306, Pmx747, Pmx732, and Pmx839 (Schall and Vardo, 2007; Vardo and Schall, 2007). When only a single allele was detected for the haploid parasite at all loci, the infection was scored as "single clone" because it is unlikely that all clones in a complex infection would carry the same allele for all 4 polymorphic loci. The microsatellite loci may not be linked to loci influencing the life history traits measured here, but they serve as a proxy for overall genetic differences among the clones. Using these same markers, experimental multi-clone infections reveal different life history traits than do single-clone infections, which suggests interaction among clones (Vardo-Zalik and Schall, 2008, 2009). Six of the donor infections (labeled as A–E, and A′) were collected at HREC and 1 clone, F, was collected at a nearby property 6.4 km from the HREC. Clones A and A′ were genotyped with identical alleles at all 4 microsatellite loci and were found in 2 infected lizards collected from within a 100-m distance at HREC. These 2 donors could have harbored the same or very similar genetic clone.

Immediately prior to initiating the experimental infections, parasitemia of each donor lizard was determined by counting asexual stages in 1,000 erythrocytes on a stained thin smear and recording number of trophozoites, multi-nucleated schizonts, and erythrocyte density in the blood, determined by using a Hausser counting chamber (Hausser Scientific, Horsham, Pennsylvania). Blood was then combined with phosphate-buffered saline (PBS) to produce a mix containing equal numbers of parasites of either a single clone or 2 clones from 2 donors. The final mixtures contained 200×10^3 sexual stage parasites in 20 µl. That is, single-clone mixtures contained 200×10^3 parasites and 2-clone mixtures contained 100×10^3 of each of the 2 clones. Previous studies showed this dose always results in a successful transfer of parasites to new hosts (Vardo-Zalik and Schall, 2009). The mix of blood and PBS was vortexed to assure cells of both clones were evenly distributed, a syringe was filled, and recipient lizards were injected intraperitoneally with 20 µl of the mixture. Thus, for each combination of 2 clones a single mix was made and injected serially into each recipient lizard to ensure uniformity of inoculum among replicates. Combinations of clones and sample sizes (number of replicates) are shown in Table I. Clone combinations including A, A′, B, and C were initiated on 17 June 2010 (Experiment I) and those with combinations C, D, E, and F were initiated 30 June 2010 (Experiment II). Not every combination of clones was possible because the small size of the donor lizards allowed only a limited volume of blood to be sampled.

Lizards were housed in outdoor, vector-proof cages (48 cm or 61 cm^3) and fed to satiation each day on crickets and mealworms. Lizards were randomly assigned to cages. Each cage was regularly rotated through each position in a 3 × 3-m array outdoors but protected from direct sun. For unplanned logistical reasons, after 60 (Experiment I) or 50 (Experiment II) days, lizards were relocated to smaller cages (63 × 38 × 43 cm high) indoors and supplied with total-spectrum lights and were also fed each day on mealworms. Blood samples were taken from a toe clip every 10 days for the first 30 days post-inoculation and every 7 days until day 50, then again every 10 days until day 80. Blood samples were used to make a thin blood smear for processing with Giemsa stain and to store a few drops dried and frozen for later genetic analysis.

Starting at day 30 post-inoculation, parasitemia of all parasite stages was determined by counting parasites seen in 1,000 erythrocytes viewed from random fields across the entire stained blood smear. We defined the infection growth rate as the maximum difference in parasitemia observed for any 2 smears divided by the number of days between those 2 samples. If the maximum parasitemia occurred at day 30, the value was divided by 30. The total productivity of the infection is the sum of parasitemia values for all samples between days 30 and 50.

Relative proportions of clones within infected lizards were determined using the methods of Vardo-Zalik et al. (2009), Ford et al. (2010), and Ford and Schall (2011). DNA was extracted from the dried blood dots using the DNeasy kit (Qiagen, Valencia, California) and the provided protocol. For each sample, 1 of 2 microsatellite markers was assessed by PCR (Ready-to-Go beads, GE Healthcare, Piscataway, New Jersey) using fluorescently labeled primers and reaction conditions previously described (Schall and Vardo, 2007). A single microsatellite locus could distinguish all combinations of the clones (Table I) except for A and A′, so this combination of clones is excluded from some comparisons. The resulting PCR product was processed through the ABI Prism® 3100 Genetic Analyzer and data were examined using GeneMapper® 3.5 software (ABI, Foster City, California). Relative proportions of the clones were assessed using the amplicon success for each microsatellite allele. Peak heights for each allele (and thus clone) were added and each height divided by the sum to give the proportion of each clone (Ford et al., 2010; Ford and Schall, 2011).

We scored relative proportion of each clone upon first patency (ability to PCR-amplify the parasite from a blood sample at typically 20 or 30 days post-inoculation), which assessed potential differences in the ability of each clone to establish in the blood. In this study, establishment success includes the ability of the parasite to survive blood transfer from one lizard host to another and then enter the blood for initial reproduction. Also, the relative proportion of each clone was followed in the infections over time (at each sample during the 80 days of the study).

Analysis was performed using JMP v8 (SAS Institute Inc., Cary, North Carolina), and R (R Development Core Team, Vienna, Austria).

RESULTS

The median rate of increase in parasitemia for all experimental infections ("growth rate") was 2.1/1,000 erythrocytes/day (range 0.2–12.8), and median total parasite production over 4 sample periods was 100 parasites per 1,000 erythrocytes (range 7–410). Total parasite production and growth rate were correlated ($r = 0.92$, $P < 0.0001$); thus, rapidly growing parasitemia typically produced the highest total number of parasites. The unplanned

TABLE I. Experimental infections of *Plasmodium mexicanum*. Given are the clone identification (A–F and A′) and number of replicates for single-clone infections and 2-clone infections. Timing of initiation of infections and movement from outdoor to indoor cages differed for the 2 experiments (see text). Also given is the proportion of trophozoites versus schizonts from each donor used to initiate the replicate recipient infections.

		2-clone				
Single clone		Experiment I		Experiment II		
ID	n	ID	n	ID	n	Percent trophozoites
A	2	AA′	3	CE	3	57
A′	2	AB	3	CF	3	67
B	2	AC	4	DE	3	68
C	4	A′B	3	DF	3	69
D	2	A′C	3	EF	3	63
E	2	BC	3	—	—	63
F	2	—	—	—	—	30
Total	16	Total	19	Total	15	

need to shift lizards from the outdoor to indoor cages opened a possible source of experimental error if the environmental change influenced the parasite life history. To test for this, the *ln* parasites/1,000 erythrocytes was regressed on days post-inoculation; 24/34 2-clone infections showed $R^2 > 0.90$, indicating that changing the environment did not alter growth rate from exponential. In all cases where $R^2 < 0.90$, the lower correlation was due to outliers early in the infection when parasitemia was low rather than to a shift coinciding with a change in housing condition. All subsequent analyses were performed using both growth rate and total parasite production, but because these 2 are tightly correlated and qualitative results matched, only the results using total parasite production are given here.

The final sample size for analysis of relative proportions in 2-clone infections was 29 lizards for all combinations. Five lizards were excluded from this analysis, which contained clones that could not be differentiated by microsatellite analysis (AA′ n = 3, which could have contained a single genetic clone), failed to amplify alleles (n = 1), or included an additional clone (n = 1), most likely 1 that was present in the recipient lizard that had been scored as not infected. Infections differed in change of relative clonal proportions over time (median = 0.10 maximum change, range = 0.04–0.37) but only 6/24 infections showed a change > 15%. Figures 1 and 2 present examples of infections with relative proportions that showed different growth rates and different degrees of change in clonal proportions. Parasite production and change in relative proportions of clones were not correlated (Fig. 3), so both the rapidly and slowly growing infections could be associated with either little or great change in clonal relative proportions. For example, 1 infection rose from 25 to 725 parasites/1,000 erythrocytes in 39 days, but both clones remained at very similar proportions throughout the infection. The necessary shift of the experimental animals from outdoor to indoor cages presented the possibility that changes in relative clonal proportions could have been driven by the change in host environment. This was not the case. Of the 9 infections with greatest change in clonal proportions, 6 showed the change before the move and 3 after. No clone effect was seen for degree of change in relative proportions (median test, $P = 0.243$).

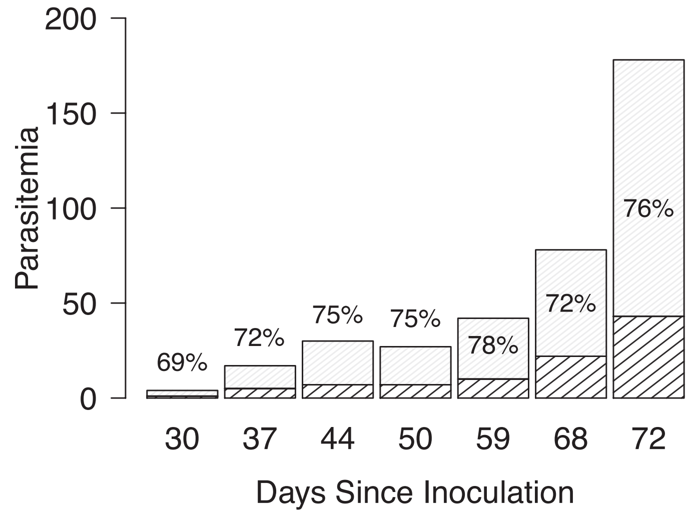

FIGURE 1. Example of an infection of *Plasmodium mexicanum* containing 2 genetically distinct clones in the lizard host's blood (*Sceloporus occidentalis*). Parasitemia is the number of parasites of all stages seen in 1,000 erythrocytes counted. Parasitemia increased rapidly in this infection but relative proportion of clones changed little (proportion of upper clone in each bar is given as percent).

When infections first became patent, relative clonal proportions often differed substantially (Fig. 4). If the clone had no influence on establishment success, each clone should have had equal numbers of infections above and below 50% at establishment. A binomial test of proportions was used to test this null hypothesis. Because there were multiple comparisons (7 clones), the *P*-value was set at 0.01 to prevent Type I errors. Clones A and C were significantly different from the null expectations ($P = 0.008$ and $P = 0.004$, respectively). Note that each clone was mixed with 2 to several other clones (Table I), so the clone effect could not be driven entirely by a specific interaction between clones A and C

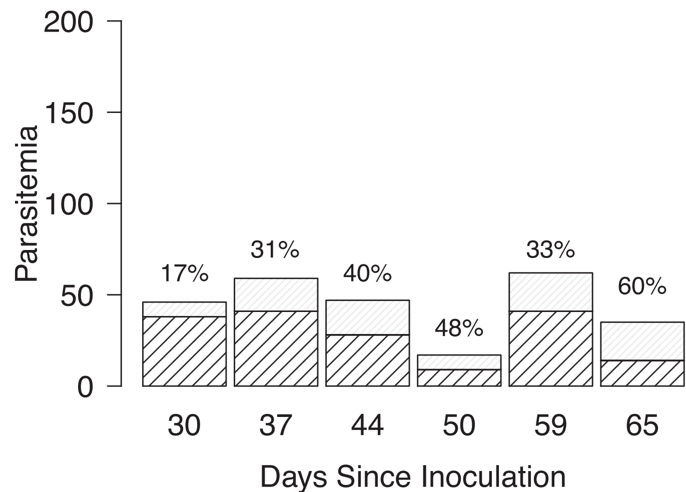

FIGURE 2. Example of an infection of *Plasmodium mexicanum* containing 2 genetically distinct clones in the lizard host's blood (*Sceloporus occidentalis*). Parasitemia is the number of parasites of all stages seen in 1,000 erythrocytes counted. Parasitemia remained low throughout the infection's history but relative proportion of the clones changed, with 1 clone increasing from 17 to 60% over the history of the infection.

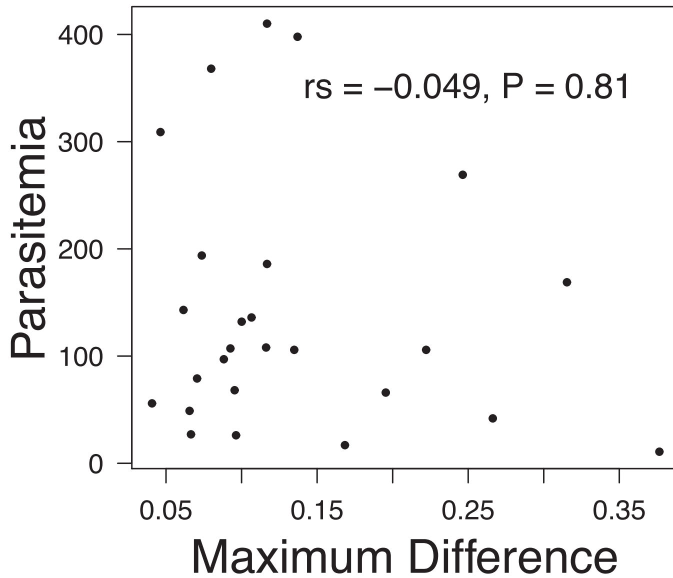

FIGURE 3. Relationship between rate of increase of *Plasmodium mexicanum* parasites in the blood of an infected lizard *Sceloporus occidentalis* and change in relative proportions of 2 coexisting clones of the parasite. Parasitemia is the sum of parasites of all stages counted in 1,000 erythrocytes counted from days 30–50 and is tightly correlated with rate of increase in parasite density over the course of the infection. Maximum difference is the maximum difference in proportions of the 2 clones measured over the course of the infection. No association was found (Spearman rank correlation given). Thus, infections with slight changes in relative proportion and those with substantial change could both be growing slowly or rapidly.

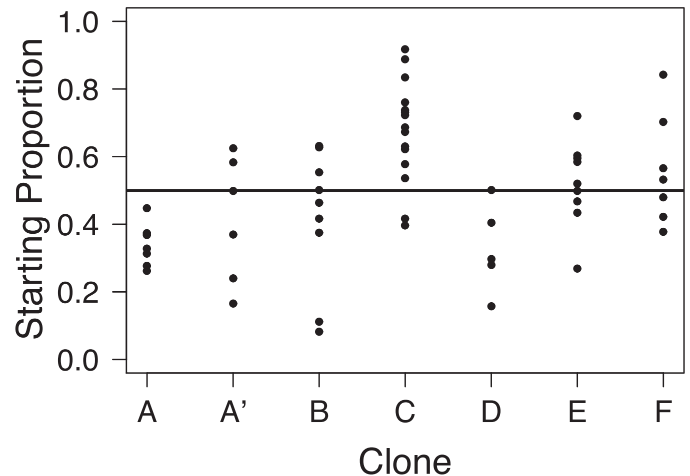

FIGURE 4. The starting relative proportion of each clone of *Plasmodium mexicanum* when in 2-clone experimental infections in its lizard host *Sceloporus occidentalis*. Horizontal line indicates equal proportions of the 2 clones. Data show results for infections carrying each of 7 clones (A–F and A′). Binomial test shows clone A with a poorer, and clone C with a better, establishment efficiency than expected by chance (see text).

nor by 1 kind of possible experimental error, a syringe effect rather than a clone effect. For example, clone C typically had higher initial proportions and this effect was found in mixes containing clones B, A, A′, and F. Success of each clone was not associated with its rate of increase when alone in an infection (correlation of parasite production vs. proportion of infections above 50% relative proportions; $P = 0.711$). A possible experimental error could be the different proportions of single-nucleus trophozoites versus multi-nucleated schizonts in the mixture inoculated. A single schizont would produce multiple daughter cells and thus have a greater influence on the early infection than trophozoites. However, all donors were similar in the proportion of schizonts except F, which had more schizonts. However, F did not display an overall greater establishment success (Table I, Fig. 4).

DISCUSSION

Molecular techniques reveal what has long been suspected for microparasites, including *Plasmodium*—that their genetic diversity is high and mixed-genotype infections are common (Walliker, 2000; Read and Taylor, 2001). This is true for the lizard malaria parasite *P. mexicanum*. Assessing a group of microsatellite markers shows there are a large number of genotypes of the parasite cycling at the California study region (Vardo and Schall, 2007), with frequency of microsatellite alleles differing among sites only a few kilometers separate (Fricke et al., 2010) and changing over years (Schall and St. Denis, 2013). These genotypes differ in important life history traits such as gametocyte sex ratio (Neal and Schall, 2010) and perhaps male gametocyte fecundity (gamete production) (Neal, 2011). Genetically mixed infections of malaria parasites also present the opportunity for complex interactions among clones. Interaction among clones appears to be present for *P. mexicanum* because complex infections tend to be more variable in growth rate and differ in several measures of virulence for the lizard host (Vardo-Zalik and Schall, 2008, 2009).

The relative proportions of clones within mixed infections of malaria parasites should also influence the outcome of inter-clone interactions, but clonal proportions are rarely quantified in ecological studies. In part, this is an outcome of the logistical difficulties in determining the relative density of 2 or more clones of a coexisting parasite. Development of real-time quantitative PCR for laboratory strains of *P. chabaudi* now allows sophisticated experiments (Cheesman et al., 2003), but for natural systems with a large number of cycling clones other methods are necessary. Verification studies of microsatellite markers for *P. mexicanum* shows that this technique is useful in measures of clonal abundance (Vardo-Zalik et al., 2009; Ford et al., 2010; Ford and Schall, 2011). Transmission success of clones moving into the sand fly vector (*Lutzomyia vexator* and *Lutzomyia stewarti*; Fialho and Schall, 1995) matches their relative proportion in the lizard host's blood (Vardo-Zalik, 2009). Thus, the relative proportion of clones determines their transmission success. Also, density of gametocytes in the lizard's blood influence overall transmission success to the vector (Schall, 2000).

We examined replicate 2-clone infections of *P. mexicanum* and found substantial variation in parasite production (64-fold range) and change in relative proportions of the 2 clones over time (3–37%; Fig. 3). Most infections, though, experienced rather little change in relative clonal proportions, a similar result to what has been seen in previous experiments with 2-clone infections (Ford and Schall, 2011). Rate of parasite increase was not correlated with change in clonal proportions. This suggests that whatever drives differences in parasite density in the blood (a combination of replication and clearance) is experienced equally by both clones

in most infections. In these infections, the establishment success at first appearance in the blood may then determine the transmission success of each clone.

We asked if there is a clone effect on both the changes in clonal proportion over time and in the initial proportions. A clone effect was seen only in the initial starting proportions; thus, clone C was more successful than expected by chance and clone A less successful. However, the overall effect was weak (Fig. 3), and most clones differed in success among replicate infections. For example, clone B dominated in some infections (up to 65% initial proportion) and failed in others (10% initial proportion). The residual variation after the clone effect is subtracted could be accounted for by experimental error in which different numbers of parasites were introduced into different hosts even though the combined cells were well mixed. Hosts also vary in the internal environment presented to the parasite. The recipient hosts were all male lizards and of the same approximate age but, nonetheless, there must be genetic and physiological diversity among lizards that could influence the outcome. Previous studies found no clonal variation for *P. mexicanum* replication rate in the blood (Vardo-Zalik and Schall, 2009; Neal and Schall, 2013), and here we found no relationship between parasite production in single-clone infections and that clone's establishment efficiency. Thus, events early in the infection that determine the proportion of clones (which is durable in most infections) remain unknown.

The results surprised us for 2 reasons. First, if relative proportions of clones determine transmission success to the vector, as shown by Vardo-Zalik (2009), why isn't there intense competitive interaction among clones and frequent shifts in relative proportions after initial establishment? Clones should compete for abundance in the blood to insure transmission success. Instead, clonal proportions typically remain constant; the pattern has been found now for several studies on *P. mexicanum*, so it appears genuine. Second, if some genotypes of parasite are better at entering a host, and then maintaining that advantage, why hasn't selection eliminated poorly performing genotypes from the population? Even a weak clone effect on establishment success should allow selection to work. Experimental studies on *P. mexicanum* show no clonal variation for several important life history traits in the vertebrate host including replication rate, maximal parasitemia, and timing in the switch to gametocyte production (Vardo-Zalik and Schall, 2009; Neal and Schall, 2013). Two traits reveal clonal variation; gametocyte sex ratio (Neal and Schall, 2010, 2013) and, as shown here, establishment efficiency. Why should genetic variation remain for such important traits? Life history studies on many species have shown complex trade-offs which maintain genetic variation for traits shown to be under strong selection (Stearns, 1992). Taking a life history approach to the study of malaria parasites should, therefore, offer insights into the complex events within infections (Eisen and Schall, 2000; Neal and Schall, 2013).

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