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ORIGINAL PAPER

Dynamics of clonal diversity in natural infections of the malaria parasite *Plasmodium mexicanum* in its free-ranging lizard host

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Abstract Within mixed-genotype infections of malaria parasites (*Plasmodium*), the number of genetic clones present is associated with variation in important life history traits of the infection, including virulence. Although the number of clones present is important, how the proportion of those clones varies over time is poorly known. Clonal proportions of the lizard malaria parasite, *Plasmodium mexicanum*, were assessed in naturally infected free-ranging lizards followed in a mark-recapture program over as long as two warm seasons, the typical life span of the lizard. Clonal proportions were determined by amplifying two microsatellite markers, a method previously verified for accuracy. Most blood samples had been stored for over a decade, so a verification test determined that these samples had not degraded. Although the environment experienced by the parasite (its host) varies over the seasons and transmission occurs over the entire warm season, 68 % of infections were stable over time, harboring a single clone (37 % of infections) or multiple clones changing only 1–12 % maximum comparing any two samples (31 % of infections). The maximum change seen in any infection (comparing any two sample periods) was only 30 %. A new clone entered three infections (only once successfully), and a clone was lost in only three infections. These results mirror those seen for a previous study of experimentally induced infections that showed little change in relative proportions over time. The results of this study, the first look at how clonal proportions vary over time for any malaria parasite of a nonhuman vertebrate host for natural infections, were surprising because experimental studies show clones of *P. mexicanum* appear to interact, yet relative proportions of clones typically remain constant over time.

Keywords *Plasmodium* · Clonal proportions · *Plasmodium mexicanum* · Mixed-genotype infections

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Introduction

Malaria parasites (*Plasmodium* spp.) cycle in their host populations as diverse arrays of genotypes (Beck et al. 2009). This conclusion emerges from studies using a variety of biochemical and molecular genetic techniques, including protein electrophoresis (Carter 1978), surface protein allele length and sequence (Felger et al. 1999), and single-nucleotide polymorphisms (Neafsey et al. 2012). Perhaps the most useful markers for studies on the parasites' genetic diversity are microsatellites, short-tandem repeats of two to six nucleotides that are highly polymorphic in repeat length; microsatellites are assumed to be selectively neutral but linked to coding loci that are important for *Plasmodium* biology (Anderson et al. 2000; Ferreira et al. 2007; Vardo and Schall 2007; Gray et al. 2013). This genetic diversity within the overall parasite population often results in mixed-clone infections both for human (Imwong et al. 2007; Zhong et al. 2007; Koepfli et al. 2011) and nonhuman (Vardo and Schall 2007; Jari et al. 2008) vertebrate hosts. Such multiclonal infections present opportunities for complex ecological effects among clones (Read and Taylor 2001). These effects include competition among clones (de Roode et al. 2003; Bell et al. 2006), clinical presentation (such as fevers and other indications of illness) based on both the diversity of the infection (Farnert et al. 1999; Branch et al. 2001) and super-infection with novel genotypes (Contamin et al. 1996), overall virulence of the infection (Taylor et al. 1998; Vardo-Zalik and Schall 2008),

replication rate of the asexual parasite cells (Vardo-Zalik and Schall 2009), exclusion of newly entering parasites, termed premunition (Smith et al. 1999; Vardo et al. 2007), transmission success into the insect host (Taylor et al. 1997), drug resistance (Wargo et al. 2007a, b; Huijben et al. 2010), and the sex ratio of gametocytes (Reece et al. 2008; Schall 2009). This summary reveals that every aspect of the biology of malaria parasites investigated to date is influenced by clonal diversity within infections.

If the number of coexisting clones is very important for the biology of malaria parasites, what is the significance of the relative proportions of those clones within an individual host? That is, mixed infections could include a single dominant clone coexisting with rare clones, or multiple clones with more even relative proportions. Assessing the relative proportion of coexisting clones for human malaria parasites is challenging. For *Plasmodium falciparum*, the number of clones appearing in the blood and their relative proportions changes over only weeks (Daubersies et al. 1996), days (Farnert et al. 1997; Farnert 2008; Bretscher et al. 2010), or even hours (Jafari-Guemouri et al. 2006). This is most likely a result of parasites sequestering in deep tissues and not appearing simultaneously in the circulating blood. *Plasmodium vivax* displays a different dynamic picture in blood cells because parasite clones emerge from the liver in successive waves (Chen et al. 2007). Thus, for these two human malaria parasites, even defining the meaning of relative proportions may be impossible. The pattern for the other human malaria parasites is not currently known. The rodent malaria parasite, *Plasmodium chabaudi*, presents a more tractable picture because relative abundance of clones can be measured in experimental infections in laboratory mice using reverse-transcription and real-time polymerase chain reaction (PCR) (Cheesman et al. 2003; Drew and Reece 2007). For example, clonal proportions appear to drive the sex ratio of gametocytes as predicted by evolutionary theory (Reece et al. 2008). Shifts in relative proportions may also indicate competition among clones, perhaps mediated in complex ways through the host immune system (Bell et al. 2006; Råberg et al. 2006).

Studies on clonal proportions of another system, a lizard malaria parasite, *Plasmodium mexicanum*, in its natural host, the western fence lizard *Sceloporus occidentalis*, present several intriguing patterns, and offer the only data for a malaria parasite in its natural, nonhuman vertebrate host. Using microsatellite markers to determine relative proportion of two to several clones in experimentally induced infections revealed that the proportions often experience very little change over time even when parasitemia rises or falls (Ford and Schall 2011; Hicks and Schall 2013). In these stable infections, the clones must be replicating or being cleared at the same rate, a pattern also observed in experimental infections of *P. chabaudi* (Cheesman et al. 2003). Other experimental infections show gradual change in relative proportions, most often when a more rare clone gradually increases, perhaps because of more rapid replication rate or lower signal to the host immune system. These data for *P. mexicanum* and *P. chabaudi*, though, are based on induced experimental infections. Thus, overall, information on clonal relative proportions for natural *Plasmodium*-host systems is scant, but the laboratory experiments argue this is an important aspect of the biology of malaria parasites.

We therefore ask here if the patterns seen in experimental infections of *P. mexicanum* occur in natural infections in free-ranging lizard hosts. In this situation, the parasite experiences shifts in its host environment over the warm season. Also, the parasite could confront sudden arrival of new genotypes when the host is bitten by additional infected vectors. We have followed lizards, *S. occidentalis*, naturally infected with the malaria parasite *P. mexicanum* in a mark-and-recapture program at a site in Northern California, USA where the parasite and its vertebrate and insect host have been under study for more than three decades (Schall and St Denis 2013). Each lizard in the study was captured, blood sample taken, and then returned to its point of capture to be recaptured multiple times. We determined both the number of clones present and their relative proportions for each capture period using two microsatellite markers. Approximately half of the lizards were followed over two warm seasons, the typical life span of the animals. Two possible general patterns within infections are (1) the number and relative proportions of clones are set early in the infection so clones reach an equilibrium set of life history traits such as replication rate or (2) clonal numbers and proportions change either gradually or abruptly, and life history traits frequently may shift over the duration of the infection. We therefore asked two questions: First, how often do new clones enter an established infection? The vectors are two species of sand flies, *Lutzyomia*, which are active at the site throughout the warm season (Schall and Marghoob 1995). Experimental evidence suggests that premunition plays a role in the transmission biology of *P. mexicanum* (Vardo et al. 2007), which suggests that most infections may remain refractory to superinfection. If so, bites by infected vectors later in the history of an infection may not lead to successful introduction of additional parasite clones. Second, relative clonal proportions change little in most experimental infections, but does this apply also to infected lizards that are free-ranging, and facing a diverse natural environment including variation in the host's diet, social status, and body temperature? If the relative proportions remain stable, what happens if a new genotype of parasite arrives in the host? This is the first study of relative clonal proportions for any nonhuman malaria parasite in its natural environment, and offers a window into the ecology of multiclone *Plasmodium* infections.

Methods

Sample collection

Fence lizards infected with *P. mexicanum* were surveyed during two mark-and-recapture studies conducted at the University of California Hopland Research and Extension Center (HREC) in Mendocino Co., CA, USA where *P. mexicanum* has been under study in its lizard host, *S. occidentalis*, since 1978 (Fricke et al. 2010; Schall and St Denis 2013). The first study was performed over the course of two consecutive warm seasons from 1996 to 1997 at one site (39.0044 N, 123.0760 W) (Eisen and Wright 2001). The second study was performed over a single warm season in 2010 and included lizards from four sites within 1 km of the first mark-and-recapture study (39.0044 N, 123.0863 W; 39.0044 N, 123.0760 W; 39.0063 N, 123.0869 W; 39.0000 N, 123.0821 W).

In both studies, lizards were collected by noosing, the animal was kept in a cloth sack until evening when blood samples were taken, and then returned to the point of capture the next morning. Each lizard was marked with a unique set of toe clips to identify individuals upon recapture (methods described in Bromwich and Schall 1986; Eisen 2000). A toe clip allowed taking a drop of blood for a thin blood smear (later processed with Giemsa stain), and several other drops were dried on filter paper and kept in a plastic bag containing silica gel beads at −20 C. The thin smears were examined at 1,000× to identify infected lizards (Fig. 1). Of the 860 lizards collected at least two times in the first mark-and recapture-study, we selected 30 lizards for analysis of clonal number and proportion and of 8 lizards sampled during 2010, 5 were chosen. Enrollment criteria for lizards to be included in the study were infected with *P. mexicanum*, a minimum of 2 weeks between first and last sample and at least three samples with dried stored blood.

Parasitemia

Parasitemia was measured as total parasites (meronts and gamonts) seen per 1,000 erythrocytes counted under 1,000×.

Genetic diversity within infections

For each blood sample taken, DNA was extracted from blood dots using the Qiagen DNeasy kit (Qiagen, Valencia, CA, USA). Two microsatellite markers were amplified (*Pmx 306* and *Pmx 747*) using conditions described by Schall and Vardo (2007). PCR products were processed on the ABI 3100 Genetic Analyzer (ABI, Foster City, CA, USA), and the resulting electropherograms were analyzed using Genemapper 3.5 software.

The vertebrate blood stages of *Plasmodium* are haploid, so each peak on the electropherogram for a single microsatellite marker could represent a single clone or two or more clones if they had the same microsatellite length allele (number of repeats of the ATT motif). For samples in which more than one allele was present at a given locus, the heights of the peaks were added, and the height of each peak was divided by the total to give the percentage of each allele, and thus each presumed clone, in the sample. This method to determine relative proportion of clones has been validated using experimental two or three clone infections (Vardo-Zalik et al. 2009; Ford et al. 2010; Ford and Schall 2011; Hicks and Schall 2013). Infections were then scored as single clone throughout the study (based on the two microsatellite markers showing a single peak or allele) or multiclone based on at least two peaks for at least one of the markers. For multiclone infections, based on the two loci, the minimum number of clones present would be the maximum number of peaks seen for either locus, and the maximum number of clones would the product of the number seen at each locus. We use the minimum number of clones here. Also tallied was the maximum change in relative proportions for any allele for either marker.

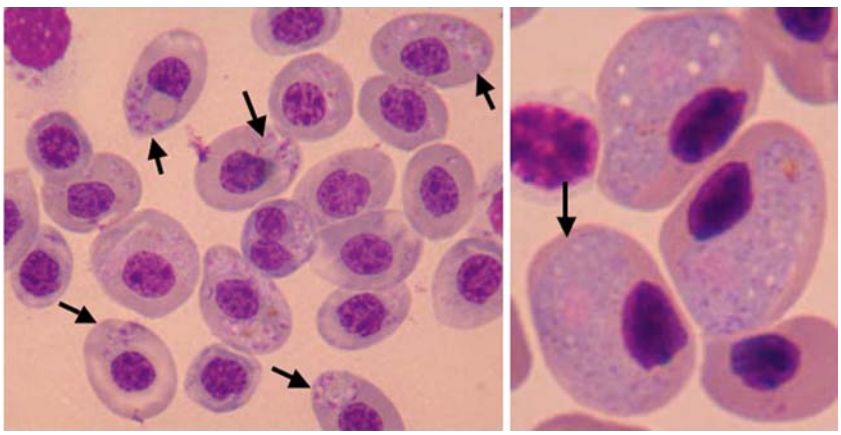

Fig. 1 Giemsa-stained thin blood smears showing *Plasmodium mexicanum* trophozoites and schizonts in a high-parasitemia infection (*left panel*) and macrogametocytes (*right panel*) in an infected fence lizard, the natural host of *P. mexicanum*

Infected lizards in which number of microsatellite alleles increased would indicate a new clone entering the host. In such cases, we wanted to insure that we had not misidentified a particular lizard. The samples were thus scored for two microsatellite markers specific to *S. occidentalis* (from Fricke et al. 2010) to ensure that the series of samples analyzed was actually collected from a single lizard, rather than a lizard being misidentified by its pattern of toe clips.

Integrity of samples

A majority of blood samples used for this study had been stored dried and frozen for more than a decade. Any degradation of DNA over that time could result in spurious results, especially for the relative proportions of clones amplified by polymerase chain reaction (PRC). Therefore, a trial was conducted in which dried blood dots from four infections scored with two alleles for loci Pmx306 and Pmx747 sampled in 2010 were submitted to several treatments that could cause DNA degradation. For each infection, one dot was kept frozen in a sealed 1.5-ml vial with or without silica gel beads. These two treatments were the control samples. Two other sets of dots were removed each day to thaw, then refrozen (11 times over a 2-week period). One set remained closed with silica gel beads, and the other was kept open without beads. For this last set of dots, water condensed from the air each day on the paper, then dried over the next several hours before the dots were placed back into the freezer. DNA was then extracted, and microsatellite loci Pmx306 and Pmx747 were genotyped as above.

Results

Integrity of stored samples

Control and the two freeze-thaw treatments produced nearly identical results for both microsatellite loci based on the peaks seen on the pherograms. Both alleles were always detected and their relative proportions differed by <2 % for the treated and control groups even after 11 freeze-thaw cycles (ANOVA of deviations among all samples, $P>>0.05$). Therefore, we conclude that long-term storage of the dried blood samples was unlikely to have resulted in degraded DNA that would alter the results of the study.

Number of clones and relative proportions over time

Of 35 infected lizards, 13 (37 %) maintained a single clone (based on the two microsatellite loci) including 7 lizards that were followed over two warm seasons (Table 1). These lizards followed over two warm seasons passed through the entire annual transmission period without gaining an additional parasite clone. An example is lizard 253 that was sampled 15 times over a 365-day period, 8 times from June through August 1996 and 7 more times in 1997, and maintained only a single scored parasite clone (one allele was seen for each of the microsatellite loci).

The 22 lizards with multiclone infections fell into four groups (Table 1). Overall, infections seen in 2 years tended to show greater change in relative proportions (Fig. 2; Wilcoxon test, $P=0.02$). Those in which the number of clones remained constant (2, 3, or 4 based on number of alleles at either locus), fell into two of those groups, based on the change in relative proportion of clones. One group (11 infections) experienced a maximum change in relative proportions of 1–12 % comparing any two samples (Table 1). Five infections showed more substantial change, from 22 to 30 % change for any two sample times. In each case, relative change agreed for both loci. We term these bimodal patterns as "stable" or "unstable" infections. Both stable and unstable infections were seen for infections followed over a long period, or even both years (Table 1).

Among stable infections, 8 were two-clone infections and 3 three-clone infections. One example of a three-clone stable infection over two warm seasons is shown in Fig. 3. This animal was first sampled in early summer the first year, then sampled five more times into the following spring. One clone was dominant during that time (~80 %) offering ample opportunity for one of the other clones to be lost by purely stochastic events, but both remained at proportions similar to those seen at first capture. Total parasitemia (shown in Fig. 3) increased during the first warm season, then dropped over the winter, to rebound the next year. Thus, although parasitemia was changing throughout the period, the three clones experienced very little measured change in relative proportions. Overall, seven stable infections showed low parasitemia (<30 parasites per 1,000 erythrocytes) that changed little over the study period, three showed an increase (three- to sixfold), including the infection shown in Fig 3, and one showed a decrease (57 to 19 parasites per 1,000 erythrocytes). Thus, the stable infections showed a range of dynamics in parasitemia.

The other 11 infections revealed an array of changes in relative proportions. Five infections kept all clones (two to four clones), with the dominant clone increasing ($N=1$) or decreasing ($N=4$). The maximum change between any two samples for any clone ranged from 22 to 30 %. All of these infections had low parasitemia (<30 parasites per 1,000 erythrocytes). One example of a four-clone infection is shown in Fig. 4, in which the minor clones fluctuate (sometimes not being detected for a sample). The parasitemia for this infection remained low (<20 parasites per 1,000 erythrocytes), so the method may miss minor clones in some samples in such a low-grade infection. A clone was lost in three infections, with one or more minor clones being lost, and all within one warm

Table 1 Summary of fence lizards (*Sceloporus occidentalis*) infected with the malaria parasite *Plasmodium mexicanum* followed during a mark-recapture program at a site in northern California. Total number of infected lizards followed was 35. Given are number of infected lizards with a single clone of parasite throughout the study, those with infections with multiple clones that showed a slight change in relative proportions of clones (<13 %) and or with significant change in relative proportions (>12 %), and infections that lost or gained a clone. Also given are years sampled, number of times the lizard was sampled and released, and the number of days between first and last capture

Infection type	Number of infected lizards	Years sampled	Range in number of samples	Mean (range) time sampled in days
Single clone	13	1996: 1 1997: 4 1996–1997: 7 2010:1	3–15	200 (17–369)
Multi clone <13 % Change	11	1996: 4 1997: 3 1996–1997: 1 2010: 3	3–13	100 (36–387)
Multiclone >12 % Change	5	1996–1997: 4 2010: 1	4–12	287 (90–371)
Clone(s) lost	3	1996–1997: 3	5–6	347 (341–351)
New clone enters	3	1997: 1 1996–1997: 2	4–7	239 (101–311)

season (example in Fig. 5). In all of the three infections in which a clone was lost, there was a concomitant drop in parasitemia, suggesting the other clones did not respond by increasing their own growth and density (total parasitemia 34 to 5, 16 to 1, and 44 to 11/1,000 erythrocytes). The apparently lost clone may well have remained, but, at a low level, not detected after the parasitemia dropped.

An invading clone was seen in only three infections, with apparent success in one (although the new clone remained at a low proportion of ~11 % in an infection with a low parasitemia of 1/1,000 erythrocytes), failure in one (Fig. 5), and an unknown outcome in the third because the entering clone was at a very low proportion (about 2 %) only at the last sample period. In all these infections, the lizard microsatellite genotypes matched for each sample indicating these were in fact the same lizard being sampled.

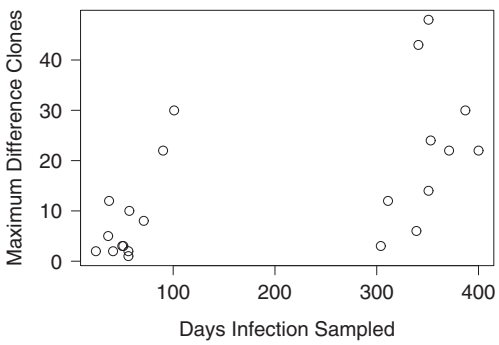

Fig. 2 Maximum change in relative proportions of clones in mixed-genotype infections of the lizard malaria parasite *Plasmodium mexicanum* by length of time a naturally infected lizard was followed in a mark-recapture program. This figure shows a tendency for a greater change to be observed for infections followed over two warm seasons; nonetheless, variation in change was similar for lizards followed for one or two seasons

Discussion

Studies on the genetic structure of *Plasmodium*, both within individual infections, and over all infected hosts at a site, are central to the understanding of malaria parasites needed for control efforts, including vaccine design (Lopez et al. 2012). Estimating the prevailing levels of multiplicity of infection (MOI) present technical challenges, but even more difficult has been determining the relative proportions of clones within mixed infections. In this first examination of clonal diversity in natural infections of a malaria parasite in a nonhuman vertebrate host, a mark-recapture program allowed determination of relative clonal proportions of *P. mexicanum* over time in free-ranging fence lizards. Data on how specific clones of a malaria parasite interact within the vertebrate host are available only for *P. chabaudi*, a parasite of African thicket rats, in

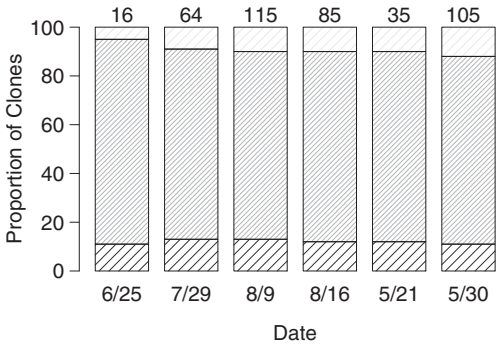

Fig. 3 Relative proportions of three clones of the malaria parasite *Plasmodium mexicanum* in a naturally infected host, the western fence lizard, at six samples taken during a mark-recapture program. Date lizard was sampled over two warm seasons is given as well as parasitemia (above bars) as parasites per 1,000 erythrocytes. This three-clone infection showed stable relative proportions of clones, with one dominant and two lower-level clones. Parasitemia was rising over the entire sample period

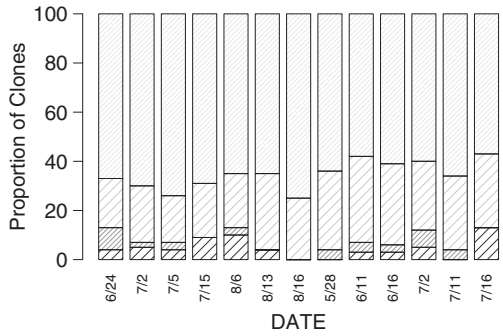

Fig. 4 Relative proportions of four clones of the malaria parasite *Plasmodium mexicanum* in a naturally infected host, the western fence lizard, at 13 samples taken during a mark-recapture program. Date lizard was sampled over two warm seasons is given. This four-clone infection showed unstable relative proportions of clones. Although relative proportions of clones varied over time, the two dominant clones remained dominant throughout the sample period

experiments using laboratory mice as the host. Although these elegant experiment are far from a natural situation (much of the ecology has been removed from these essentially ecological experiments), the results show intense interaction among clones, especially competition (de Roode et al. 2003; Bell et al. 2006). Therefore, the results for experimental infections of *P. mexicanum* in its natural host are surprising. Most often, the relative proportions of clones first seen when the parasite density becomes patent in the blood are maintained over time (Ford and Schall 2011; Hicks and Schall 2013; Vardo-Zalik et al. 2009). There is a weak genotype effect on initial establishment success in the blood for each clone, but no sign of competition over time (Hicks and Schall 2013). Our mark-recapture study sought to determine if a similar pattern emerges for lizards that were exposed to environmental variation. Also, we asked if new clones of parasite enter an already infected host (due to additional bites of infected vectors), and if entry of a new clone causes shifts in relative abundance of the original parasite genotypes. The lizards were followed as long as two warm seasons, which is close to the typical life span of *S. occidentalis* (Eisen 2001), so the results can be taken as reflecting most or all of the duration of a *P. mexicanum* infection in its vertebrate host.

P. mexicanum parasites must experience significant environmental variation within the lizard blood. Over the course of the warm season from April through September when lizards are active at the field site, the environment dries (rains fall almost entirely during the autumn and winter), and daytime temperatures increase (Schall 1982). The lizard's reproductive period is in the early warm season when both males and females expend stored fat from the previous late season (Schall 1982), and social interactions among males are frequent (Schall and Sarni 1987). For both genders, reproductive hormones must vary over time; for example, the testes in males are large in early season, then decline in mass by September (Schall 1982). Infection with *P. mexicanum* itself alters the lizard host, including changes in social status, hormone picture, and reproductive success (Dunlap and Schall 1995; Schall 1996). Overall, this argues that the hosts will vary in their diet, social status, blood hormone levels, and thermal environment over weeks, months, and perhaps between years. Thus, even if competition among clones is not intense, changes in the host environment could allow shifts in relative success of parasite genotypes within the blood.

Although each infected lizard presents its own story (as is apparent from the case stories described in the "Results" and shown in Figs. 3, 4, 5, and 6), several general patterns emerge from the study. First, entry of new genotypes of parasites into already infected lizards was uncommon, even for lizards followed over two warm seasons. Only 3 of 35 lizards showed entry of a new clone, and only in one was the entry clearly successful. Density of the sand fly vector (two species of *Lutzomyia*) is highest late in the warm season during August, but transmission occurs from April through September, based on appearance in the lizards of apparently new infections consisting only of trophozoites (Bromwich and Schall 1986; Schall and Marghoob 1995). Prevalence of infection in the

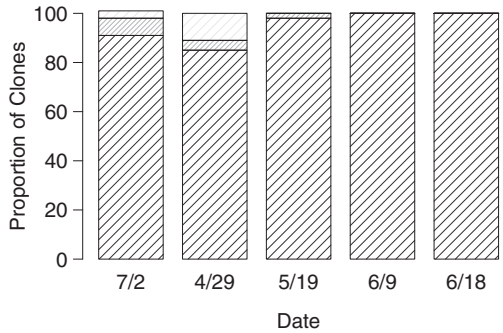

Fig. 5 Relative proportions of three clones of the malaria parasite *Plasmodium mexicanum* in a naturally infected host, the western fence lizard, at five samples taken during a mark-recapture program. Date lizard was sampled over two warm seasons is given. All clones were seen in both seasons, but the two minor clones were lost from the infection by June of the second year

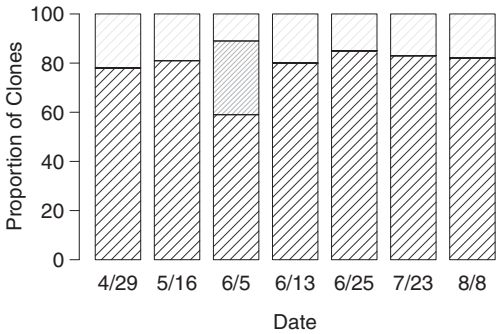

Fig. 6 Relative proportions of three clones of the malaria parasite *Plasmodium mexicanum* in a naturally infected host, the western fence lizard, at seven samples taken during a mark-recapture program. Date lizard was sampled over one warm season is given. A new clone entered this two-clone infection, but was quickly lost

years when most of the lizards were sampled was ~25 % (Schall and St Denis 2013), so during any warm season, about 9 of any 35 lizards should have gained a new clone. This result hints that established clones exclude entry of new parasite genotypes, a process termed premunition for *Plasmodium*. Such premunition has been experimentally demonstrated for *P. mexicanum* (Vardo et al. 2007).

The second pattern was the lack of major shifts in clonal proportions in almost all infections. Even for infected lizards sampled over two warm seasons, the infections scored as stable shifted <13 % for any clone comparing any two sample periods. For the infections scored as unstable, the change was only 22–30 %. Previous validation experiments compared the actual relative proportions of clone DNA entering the genetic analyzer instrument with the measured proportions, and the results matched closely, with an empirically determined 95 % confidence interval of ~±10 %. Thus, combining all mixed-clone infections shows a likely maximum shift in proportion of any two clones over the entire observation period of ~0–30 %. For three infections that lost a clone, it was always the clones with low relative proportions that were lost, so there was never a major shift in proportions that resulted in a dominant clone leaving the infection.

Combining all these results shows the most typical pattern is for stability in natural infections (including the single-clone infections as stable). This is surprising because experimental studies, with manipulated infections of malaria parasites, argue that coexisting clones interact. For example, the best experimental data on events in mixed-clone *Plasmodium* infections come from the *P. chabaudi* rodent malaria laboratory model, and reveal such competitive interactions among parasite genotypes (for example, Bell et al. 2006). Experimental infections of *P. mexicanum* show a more complex story. Single-clone infections are similar in growth rates, final parasitemia, and gametocyte production. Mixed-clone infections show great variation, with some similar to single-clone infections, and others showing higher production of both asexual cells and gametocytes (Vardo-Zalik and Schall 2009). Experimental infections also resist entry of new clones or premunition (Vardo et al. 2007), which would account for the rare entry of new clones into the natural infections seen in this study.

If clones seem to interact in some, but not all experimental infections, why do they rarely change in relative proportions in both experimental and natural infections? Another important life history trait for *P. mexicanum* also differs for natural and experimental infections, the gametocyte sex ratio. Sex ratio tends to be less female biased for experimental mixed-clone vs. single-clone infections, but not for natural infections (Neal and Schall 2014). The experimental infections for both rodent and lizard parasites may generally differ from natural infections in an ecologically important aspect. Natural mixed-clone infections may be composed of genotypes that often occur together, in part because they travel as a group within the vector, and because extremely negative interacting clones would not succeed over time together. For *P. mexicanum*, clones in the vertebrate blood become established in the vector and even in the same relative proportions (Vardo-Zalik 2009). The experimental infections could include genotypes that rarely occur in nature; indeed, the rodent malaria experiments mix clones known to differ in important life history traits. This proposal argues that clones in natural *Plasmodium* infections do not represent a random assembly from the overall genetic diversity in the parasite metapopulation.

Last, why does the likely change in host environment over time not affect the parasite to cause changes in clonal proportions? It is possible that the parasite has the ability to buffer such environmental changes. For example, experimental manipulation of the lizard body temperature does not alter the rate of asexual replication of *P. mexicanum* (Schall 1990) even over a 12° range. This remarkable thermal buffering ability may be matched by other methods to deal with physiological shifts in the lizard host.

Read and Taylor (2001) opined that the study of mixed-genotype parasite infections would offer important and surprising insights into the biology of parasites. *Plasmodium* was their own research focus. We agree, and offer as evidence the surprising outcome of both experimental manipulations and this new look into natural infections for *P. mexicanum*.

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Ethical standards The work reported here complied with all current laws of the country in which the research was conducted, and under an Animal Care and Use protocol approved by the University of Vermont and University of California.

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