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Clonal diversity of a malaria parasite, *Plasmodium mexicanum*, and its transmission success from its vertebrate-to-insect host

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ABSTRACT

Infections of the lizard malaria parasite *Plasmodium mexicanum* are often genetically complex within their fence lizard host (*Sceloporus occidentalis*) harbouring two or more clones of parasite. The role of clonal diversity in transmission success was studied for *P. mexicanum* by feeding its sandfly vectors (*Lutzomyia vexator* and *Lutzomyia stewarti*) on experimentally infected lizards. Experimental infections consisted of one, two, three or more clones, assessed using three microsatellite markers. After 5 days, vectors were dissected to assess infection status, oocyst burden and genetic composition of the oocysts. A high proportion (92%) of sandflies became infected and carried high oocyst burdens (mean of 56 oocysts) with no influence of clonal diversity on these two measures of transmission success. Gametocytemia was positively correlated with transmission success and the more common vector (*L. vexator*) developed more oocysts on midguts. A high proportion (~74%) of all alleles detected in the lizard blood was found in infected vectors. The relative proportion of clones within mixed infections, determined by peak heights on pherograms produced by the genetic analyser instrument, was very similar for the lizard's blood and infections in the vectors. These results demonstrate that *P. mexicanum* achieves high transmission success, with most clones making the transition from vertebrate-to-insect host, and thus explains in part the high genetic diversity of the parasite among all hosts at the study site.

1. Introduction

The life cycle of malaria parasites (*Plasmodium* spp.) requires transfer of gametocyte cells from the blood of an infected vertebrate host (squamate reptile, bird or mammal) to a biting insect vector (sandfly or mosquito) (Fialho and Schall, 1995; Vickerman, 2005), where sexual recombination of the parasite occurs (Baton and Ranford-Cartwright, 2005). Transmission success of the parasite from vertebrate to vector is influenced by gametocyte density in the blood (Mackinnon and Read, 1999; Schall, 2000), sex ratio of male and female gametocytes (Robert et al., 1996), specific genotypes of the parasite clones replicating in the vertebrate host (de Roode et al., 2005a,b), and vector genotype (Beier, 1998). The genetic diversity of parasite clones within the vertebrate host could also be important for the transmission success of *Plasmodium*, both overall for an infection and for the individual genotypes in the infection, but such processes remain poorly understood (but see Taylor et al., 1997a,b; de Roode et al., 2005a,b).

Cross-sectional surveys of human malaria parasites, *Plasmodium falciparum, Plasmodium vivax* and *Plasmodium malariae*, (Anderson et al., 2000; Leclerc et al., 2002; Cui et al., 2003; Imwong et al., 2006; Bruce et al., 2007) and data on malaria parasites of non-human vertebrate hosts (Vardo and Schall, 2007) reveal that infected vertebrate hosts commonly harbour more than one genotype of parasite, and such multi-clonal infections are more common where transmission intensity is high. The high frequency of multi-clonal infections therefore suggests two questions regarding the role of clonal diversity for the overall transmission biology and population genetics of *Plasmodium*. Firstly, do all clones successfully make the transition from vertebrate-to-insect? The small amount of blood taken by a vector may not include mature gametocytes of all clones within that infection, and not all genotypes that are present in the blood meal may successfully mate and complete sporogony. Thus, there could be a bottleneck reducing clonal diversity during transmission. Chronic failure of all clones to make this transition into vectors could have broad consequences for the population genetics of *Plasmodium*. Second, does clonal diversity (single-clone versus mixed-clone) influence the overall transmission success of the parasite (de Roode et al., 2003, 2005b)? Transmission potential could be reduced within mixed-clone infections if overall gametocytemia for each genotype is reduced or if intraspecific competition for establishment within the vector exists (de Roode et al., 2003, 2005b). In contrast, overall transmission potential may increase if infection gametocytemia is higher for mixed-clone infections (Taylor et al., 1997a,b; Vardo-Zalik and Schall, 2009).

This study sought to determine how the genetic diversity of clones in the vertebrate host's blood influences transmission

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success of *Plasmodium mexicanum*, a parasite of fence lizards, *Sceloporus occidentalis*, and sandflies, *Lutzomyia vexator* and *Lutzomyia stewarti*, in northern California, USA (Fialho and Schall, 1995). The life cycle is similar to that of other *Plasmodium* species, with the exceptional use of a sandfly vector rather than a mosquito. Recently characterised microsatellite markers allow identification of parasite genotypes (Schall and Vardo, 2007; Vardo and Schall, 2007) and show that a high proportion of natural infections are multi-clonal (from 50% to 85% depending on which of the five annually sampled sites at the field station is considered). Experimental infections are readily established that vary in clonal diversity (Vardo-Zalik and Schall, 2008, 2009), and experimental feeds by sandflies produce oocysts that can be counted (Fialho and Schall, 1995; Schall, 2000) and genotyped (see Section 2, Section 2.2). Replicate experimental infections of *P. mexicanum* with known numbers of parasite genotypes in the natural vertebrate host species were established, and both overall transmission success and transmission success of individual clones into the sandfly vectors were measured. Transmission success of individual clones has been followed for *P. falciparum* in natural human infections (Huber et al., 1998) and experimental infections of the rodent malaria parasite *Plasmodium chabaudi* in laboratory mice (Taylor et al., 1997a,b), but to my knowledge this is the first experimental study to focus on genetic diversity and transmission success of a non-human malaria parasite in its natural vertebrate and insect hosts.

2. Materials and methods

2.1. Experimental infections and parasite genotyping

The study was conducted at the University of California Hopland Research and Extension Center, near the town of Hopland, Mendocino County, California, USA (Schall, 1996). Naturally infected lizards were captured during May 2005 from five annually sampled sites (Vardo-Zalik and Schall, 2008, 2009) to serve as blood donors to initiate experimental infections. Blood was obtained from a toe clip to make blood smears (treated with Giemsa's stain and examined at 1,000×) and dried blood dots on filter paper for molecular analysis. Infected lizards were identified by scanning blood smears. Donor infections were chosen that had high parasitemia of asexually replicating meront stages (> 25 meronts per 1,000 erythrocytes). In early May, few infections presented high meront densities, so of the sample of 312 lizards, only seven suitable donors were identified. *Sceloporus occidentalis* are small lizards so only a small number of recipient infections could be initiated from a single donor.

DNA from infected lizards was extracted using the Qiagen DNeasy extraction kit, following the manufacturer's protocol. The parasites from the donor lizards were genotyped for three microsatellite markers (Pmx306, 747 and 732) using PCR primers and conditions presented in Schall and Vardo (2007) (Table 1). Labeled PCR product was run on an ABI Prism genetic analyser and results analysed using GeneMapper software (ABI). Of the seven donors, only two contained a single-clone of the parasite (one allele per marker). Previous trials using six markers found that the three used here were efficient at revealing such single-clone infections. For the other donors, I estimated the clonal diversity of infections as the maximum number of alleles observed for any of the three microsatellite markers (two to four clones). This estimate is widely used and gives an unbiased estimate of the minimum number of clones in an infection (Anderson et al., 2000; Ferreira et al., 2002, 2007; Bogreau et al., 2006; Bruce et al., 2007; Vardo and Schall, 2007).

To create replicate recipient infections with known numbers of clones, 80 non-infected adult male lizards (snout-vent length >54 mm) were captured from two sites where malaria has been absent for the past 30 years (J. Schall, personal communication). Smears were examined at 1,000× to view at least 10,000 erythrocytes and a sensitive PCR-based protocol that detects extremely weak infections (as low as one parasite per 1 million erythrocytes) (Vardo et al., 2005) confirmed that the recipient lizards were not already infected with *P. mexicanum*. Treatment groups, each with 10 recipients, with differing clonalities (one, two or three or more clones [3+]) and genotype combinations (a, b, or c) were as follows: two single-clone groups (1a and 1b), three two-clone groups (2a–c), and three multi-clone groups (with three or more clones; 3–3c) (Table 2).

Infections were initiated following the protocol given in Vardo-Zalik and Schall (2008, 2009). Recipient lizards were infected with blood from one or two different donor infections (Tables 1 and 2). Briefly, blood was taken from each donor lizard and quickly mixed with PBS. Twenty microlitres of the blood–PBS mixture (containing approximately 200×10^3 asexual parasites) was injected i.p. into each recipient lizard. The number of parasites injected remained constant across all treatment groups regardless of clonality; for mixed-donor treatments, each donor contributed an equivalent number of parasites to the mixture, resulting in the final count of 200×10^3 asexual parasites in 20 µl of the blood–PBS mixture.

Lizards were housed by replicate in eight outdoor cages suspended from clotheslines and shaded under a tarp. Cages were moved weekly within the array. Lizards were fed daily to satiation on mealworms or crickets and on days where the ambient temperature exceeded 27 °C, were showered with cool water every 2 h from 1300 to 1900 h.

Table 1
Seven donor infections of *Plasmodium mexicanum* and their respective numbers of clones (one to four clones per infection). Alleles and clonality were assessed via three microsatellite loci (Pmx306, 747 and 732; Schall and Vardo, 2007). The minimum number of clones within an infection (estimated via the microsatellite locus with the most alleles for that infection) is presented. Alleles at each locus are presented in reference to repeat length, with the first letter/number representing the shortest repeat (306 A–H; 747 1–6; 732 a–f).

Donor	Number of Clones	Pmx306	Pmx747	Pmx732
I	1	H	4	c
II	1	G	4	c
III	2	A C	4	c f
IV	2	D F	6	d
V	2	D	4 5	a
VI	3	A D G	2 4	b c f
VII	4	B D E H	1 3	e f a

Table 2
Treatment groups for infections with differing clonalities and genotypes of the malaria parasite *Plasmodium mexicanum*. Treatments are represented by the number of clones (1–3, with 3 including infections with three or more clones of the parasite) and replicate treatment (a–c, each with differing genotype combinations). All groups have 10 individuals. Alleles and clonality were passed via three microsatellite loci (Pmx306, 747 and 732; Schall and Vardo, 2007). The minimum number of clones within an infection (estimated via the microsatellite locus with the most alleles for that infection) is presented. Alleles at each locus are presented in reference to repeat length, with the first letter/number representing the shortest repeat (306 A–H; 747 1–6; 732 a–f).

Treatment	Donor(s)	Number of clones	Pmx306	Pmx747	Pmx732
1a	I	1	H	4	c
1b	II	1	G	4	c
2a	III	2	A C	4	c f
2b	IV	2	D F	6	d
2c	V	2	D	4 5	c
3a	VI	3	A D G	2 4	b c f
3b	VI + VII	6	A D G B E H	2 4 1 3	b c f e a
3c	II + VII	5	B D E H G	1 3 4	c e f a

Blood samples were taken 1 month after injection and also on the day of a transmission feed to estimate gametocytemia (the number of mature male and female gametocytes within an infection) and gametocyte sex ratio (proportion microgametes, or males, out of 100 gametocytes counted). Lizards in recipient group 1b were bled an additional two times before the 1 month mark, each time having approximately 2 μl of blood taken. This additional blood sampling should not bias the growth of the parasite, but an additional two drops of blood from each of the other treatment lizards were taken at the 1-month bleed to account for this. Clonality within each infection on the day of a transmission feed was assessed at three polymorphic microsatellite loci (Pmx306, 747 and 732; as described above) after the experimental period (Table 2).

2.2. Sandfly collections, transmission experiments and genotyping of oocyst population

Sandflies were captured nightly from July 8 to August 26 from 2200 to 2400 h in funnel traps as described by Schall (2000) at sites where *P. mexicanum* has been absent for the past 30 years (J. Schall, personal communication). Flies were then transferred into mesh cages, the cages wrapped in a plastic bag to retain moisture and placed inside a 26 °C incubator. After 24 h in captivity, the sandflies (10–20 at a time) were allowed to feed on an infected lizard randomly chosen from a treatment group, at room temperature. Fly catches fluctuated nightly, so the number of flies feeding on each lizard varied during the experiment. A cotton mask was placed over the lizard's eyes and head to ensure the lizard would not feed on the flies. The cage was checked periodically throughout the 24 h feeding period. Fed flies were removed and transferred to a jar in the incubator with a sucrose soaked cotton ball placed on top that was changed daily. Any unfed flies were discarded.

After 5 days, flies were dissected in dH_2O, the species of sandfly recorded (*L. vexator* or *L. stewarti*) and midguts examined for oocysts using the methods of Fialho and Schall (1995). The flies were dissected on day 5 rather than day 9 as per Fialho and Schall (1995) to minimise bursting mature oocysts. If infected, the number of oocysts per midgut was counted and the gut stored in a 2 ml vial with 100% ETOH for genetic analysis.

Midguts stored in ETOH were amplified for two (Pmx306 and 747) or three (Pmx306, 747, 732) microsatellite markers to determine the proportion of alleles within each infection (above) that contributed to the oocyst population. By day 5, oocysts were obvious on midguts and the blood meal had been digested. Thus, only clones that had become established in the vector contributed to the results. ETOH was allowed to evaporate out of the vials by leaving the vials open, covered with filter paper, within a fume hood. After 24–48 h, midguts were dry and their DNA extracted using the Qiagen DNeasy extraction protocol. The DNA was then used to amplify the genotypes present in the midgut population for two or three microsatellite markers (Pmx306, 747 and 732; as described above).

2.3. Relative abundance of parasite genotypes

Relative abundance of parasite genotypes within each lizard and the fed sandflies was scored using the peak heights for each allele from the GeneMapper pherograms. A study that altered the relative amount of DNA of different clones found that peak heights on the pherograms matched the relative proportions of each clone's DNA in the extraction (Vardo-Zalik et al., 2009). Therefore, the relative peak heights allowed determination of whether the proportions of each clone within each lizard matched those in the vector. Due to the vagaries of the Prism instrument's function (ABI manual, 2004), the peak heights were not used to estimate actual density of the parasites in lizard and vector, but only to determine whether the density in the lizard matched that in the vector.

To statistically test the differences in relative proportions of alleles within the lizard and sandfly, a χ^2 analysis was performed comparing the observed number of times the proportions matched in each host with the number of times this would be expected by chance. For this analysis, the most polymorphic marker, Pmx306, was chosen and only samples where all clones transmitted successfully to the sandfly vector were included. The relative density of each clone within an infection was gauged by the peak height of each allele on the pherogram. Alleles were given a value (1, 2 or 3) depending upon their height in relation to each other. No alleles were regarded as equal to each other in peak height. The number of different outcomes possible for each clonal group was used to estimate the expected value. Samples with two clones have two different allelic combinations that could occur, while samples with three clones have six. For example, a two-clone infection could show A > B or A < B; again, there will be no A = B because the relative density of alleles, even alleles with very similar peak heights, were considered different. Expected values were calculated by multiplying the number of samples in each clonal group by the likelihood of observing a certain order (either 1/2 or 1/6).

2.4. Data analysis

To examine the effects of clonality on oocyst burden, infectivity to the sandfly vector and gametocytemia, nested ANOVAs were used with clonal group (1, 2 or 3+, with 3+ including all infections with three or more clones) as the main effect and replicate treatment groups (a–c) nested within clones. Gametocytemia on the day of the feed (# gametocytes observed in 1,000 red blood cells) was $\mathrm{Log}_{10}(n+1)$ transformed and oocyst burden 1/3 root transformed. Non-parametric Kruskal–Wallis tests were used when the assumptions of ANOVA could not be met.

All procedures followed an animal care and use protocol approved by the University of Vermont Institutional Animal Care and Use Committee.

3. Results

3.1. Vector feeding and infection gametocytemia

Sandfly feeds were conducted on 29 infections (11 single-clone, 9 two-clone, and nine multi-clone '3+') over a 36-day period. One lizard in multi-clone treatment group 3a was fed upon twice, but the feeds were considered independent treatments because 8 days had passed between vector feedings. Sandflies dissected were 131 *L. vexator*, 30 *L. stewarti* and three unknown (Table 3). Thirteen sandflies (8% of total) did not become infected, so were excluded from analysis on oocyst burden (148 sandflies remaining). Vector sample sizes per treatment for oocyst analysis was 58 for single-clone infections, 56 for two-clone infections and 34 for multi-clone infections (three to four clones established; Table 3).

Gametocyte sex ratio for the 29 infections did not differ between clonal groups or replicate treatments (Kruskal–Wallis, $P = 0.43$ and 0.35). The distribution of gametocytemia (gametocytes per 1,000 erythrocytes) on the day of a transmission feed also did not differ across treatment groups or replicates ($F_{7,21} = 0.25$, $P = 67$ and $P = 0.95$; Table 4).

3.2. Sandfly infectivity, transmission success and oocyst burden

Infectivity to the sandfly was not affected by clonality, nor day of transmission feed, but was influenced by replicate treatment (χ^2 test, $P = 0.999$, $P = 0.744$ and $P = 0.0003$, respectively). However,

Table 3
The number and species of *Lutzomyia* sandflies that fed on lizards infected with *Plasmodium mexicanum* from each treatment group, together with the proportion of the flies that became infected for each replicate. Treatments are represented by the number of clones (1–3, with 3 including infections with three or more clones of the parasite) and replicate treatment group (a–c, each with differing genotype combinations). The number of sandflies fed (the number of flies that did not become infected) is given. The three 'unknown' sandflies were not included in the analyses as they could not be properly assigned to one of the two species.

Treatment	Number of sandflies			Proportion flies infected
	Lutzomyia vexator	*Lutzomyia stewarti*	Unknown	
1a	31 (2)	9 (3)	0	0.80
1b	20	3	0	1.00
2a	14 (2)	4	1	0.89
2b	12	4	1	1.00
2c	21	3	1	1.00
3a	28 (3)	6	0	0.92
3b	3 (3)	0	0	0.00
3c	2	1	0	1.00

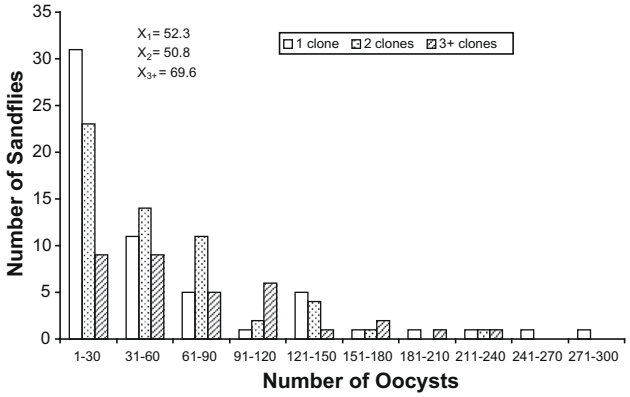

Fig. 1. Transmission success of *Plasmodium mexicanum* infections consisting of one, two or three or more clones. Transmission success was measured as the number of oocysts that developed on the sandfly vector's midgut by day 5 post-feed. The mean number of oocysts per treatment is given; transmission success was not significantly different between clonal groups.

this result was largely influenced by one treatment group (3b) with only three sandflies, all of which did not become infected (Table 3). Removing this group from the analysis, the replicate effect remained (χ^2 test, $P = 0.038$).

Oocyst burden for the infected midguts ($n = 148$) ranged from 1 to 276 per midgut, with an overall mean of 56 per midgut. The number of oocysts did not differ among treatment groups (Fig. 1; Table 4) but replicate treatment group influenced oocyst burden ($F_{6,141} = 7.3$, $P = 0.82$ and $P = 0.0001$, respectively), with groups 1a, 2b and 3c having lower burdens.

Gametocytemia on the day of sandfly feeding influenced transmission success, with more gametocytes associated with higher oocyst burdens ($F_{23,\ 121} = 2.79$, $P = 0.0003$). Replicates with highest gametocyte counts were also those with higher oocyst burdens (replicates 1b, 2a, 2c and 3a; Table 4). Fly species influenced transmission success ($F_{23,121} = 2.79$, $P = 0.043$), with higher oocyst burdens for *L. vexator*; this was not influenced by differences in infection gametocytemia ($F_{23,121} = 2.79$, $P = 0.259$).

3.3. Genetic makeup of oocyst samples and relative abundance of genotypes

A random sample of 75 midguts was chosen to determine the transmission success of clone numbers and specific genotypes from lizard to sandfly (15 single-clone, 33 two-clone and 27 multi-clone [three to four clones] infections). For these three groups, the number of oocysts per midgut did not differ ($F_{2,72} = 0.70$, $P = 0.49$). For the 75 vectors, and for the three microsatellites, only a single allele was not detected in the lizard, but appeared in a single sandfly. In most of the two-clone and multi-clone infections (using the minimum clones rule), all parasite genotypes in the lizard were represented in the sandfly after the oocysts developed (Fig. 2a; 76% and 74%, respectively). Examination of the marker with greatest allelic diversity (Pmx306), shows 95% of the two-allele infections passed both alleles to the vector, and 79% of the multi-clonal infections passed all alleles (Fig. 2b).

Pherogram peak heights for two microsatellite markers were compared for each lizard and infected sandfly pairing to determine whether the relative density of the parasites in the vertebrate was related to relative density in the vector. For Pmx306, lizards harboured two, three or four clones; the three and four-clone groups were combined. The relative peak heights were the same for 62% of the sandfly/lizard pairings; all but two occurrences of different relative peak heights were in infections with three to four alleles present (Fig. 2c). In the 18 two-clone infections, the relative density of alleles found in the vector matched that of the lizard in 16 of the pairings, significantly higher than the nine times expected by chance ($\chi^2 = 5.44$, $P < 0.05$). Similarly, of the 19 three to four-clone infections, seven pairings were the same, while only three were expected by chance alone. Combining all multi-clonal infections (two to four clones), exact matches were found in 23 of the 37 cases (expected = 11; $\chi^2 = 13.09$, $P < 0.05$).

4. Discussion

Multiple clones within an infection of a malaria parasite could influence overall transmission success from vertebrate-to-insect hosts if clones interact, either positively or negatively, resulting in altered investment in gametocytes, or gametocyte sex ratio (Taylor et al., 1997a; Taylor and Read, 1998; Eisen and Schall, 2000; Ferguson et al., 2003; de Roode et al., 2005b; Reece et al.,

Table 4
Summary data for seven treatment groups of *Plasmodium mexicanum* infections with differing clonalities and clonal combinations. Treatments are represented by the number of clones (1–3, with 3 including infections with three or more clones of the parasite) and replicate treatment (a–c, each with differing genotype combinations). Microsatellite genotypes of infections on the day of the transmission experiment, with the lowest letter/number representing the smallest allele, are given (see Table 1). The back transformed values for both gametocytemia and oocyst burden are shown. For sex ratio of gametocytes, the median percent of microgametes (males) observed is given.

Treatment	Alleles present on day of feed			Gametocytemia				Oocyst burden		
	Pmx306	Pmx747	Pmx732	Number of infections	Mean (S.D.)	Range	Sex ratio	Number of midguts	Mean (S.D.)	Range
1a	H	4	c	7	5.7 (7.3)	0–19	0.28	35	24.7 (36.3)	1–163
1b	G	4	c	4	7.8 (5.7)	2–14	0.33	23	95.4 (80.2)	2–276
2a	A C	4	c f	3	111.7 (17.7)	0–32	0.13	16	59.9 (48.1)	1–160
2b	D F	6	d	2	6.5 (9.2)	0–13	0.17	16	26.1 (33.6)	1–144
2c	D	4 5	a	4	11.5 (18.4)	0–39	0.55	24	61.2 (49.2)	2–215
3a	A D G	2 4	b c f	7	9.1 (11.0)	2–32	0.20	31	74.2 (56.5)	2–216
3c	B D E H G	1 3 4	c e f a	1	2	2	0	3	22.0 (30.5)	1–57

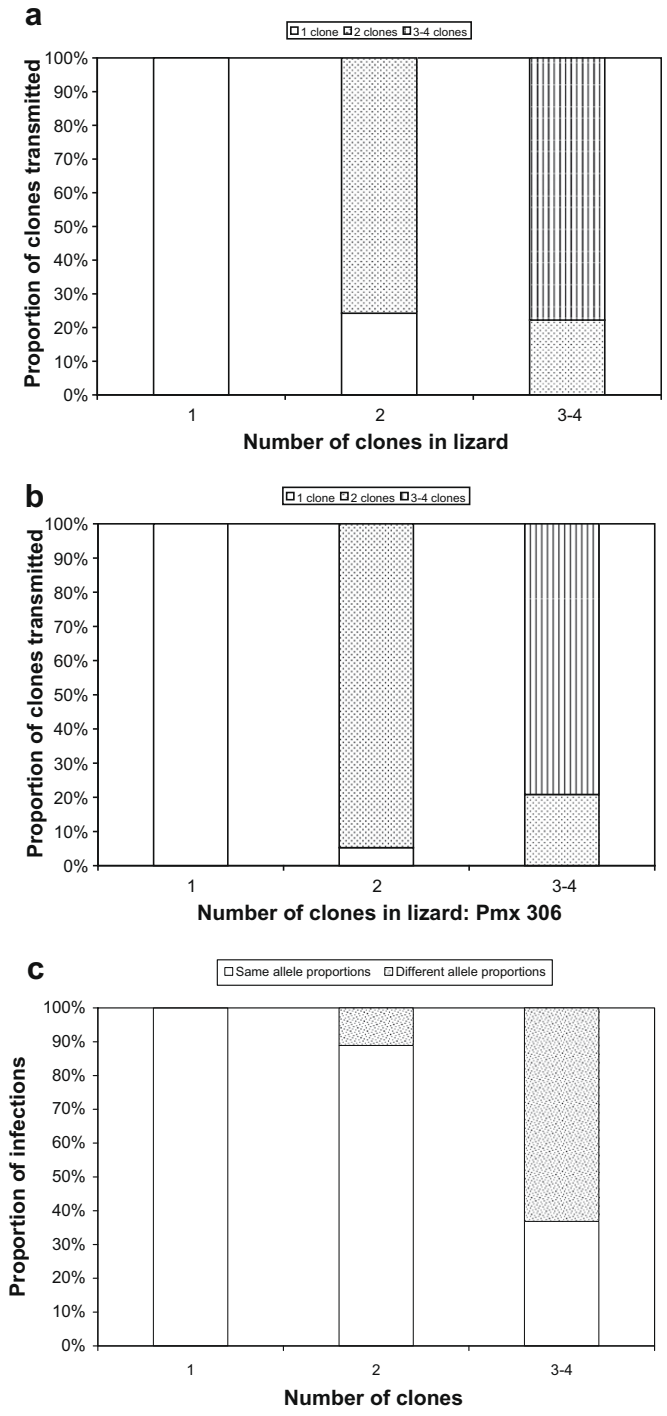

Fig. 2. Transmission success of genotypes from multi-clone infections from the lizard to the sandfly vector. Seventy-five infected midguts were analysed at two to three microsatellite makers. (a) Success of multiple clones transmitted to the sandfly vector over two to three markers. (b) Success of individual alleles at locus Pmx 306 in transmitting to the vector. In both instances, the majority of infections passed on all alleles/clones. (c) The relative density of alleles in each the lizard and the sandfly vector pairing were compared. When infections carried two clones, the relative proportions in lizard and sandfly were similar; however, when infections consisted of three or more clones, the relative proportions varied.

2008; Vardo-Zalik and Schall, 2009). The relative success of each clone could also be influenced by a variation in the rate of maturation of gametocytes (Taylor et al., 1997a,b). The present study examined the transmission efficiency of *P. mexicanum* from its lizard host into two species of sandfly vectors.

Overall transmission efficiency of *P. mexicanum* was high in the experiments, with infected sandflies carrying a mean of 56 oocysts on their midgut. Of 164 vectors fed on lizards with known numbers of clones, only 8% did not become infected, with no influence of clonal diversity on transmission success. Also, infections harbouring one-clone versus multiple clones produced similar numbers of oocysts in vectors. Although mixed-clone *P. mexicanum* infections tend to yield higher maximum densities of parasites, including gametocytes, in the lizard host's blood (Vardo-Zalik and Schall, 2009), no influence of clonal diversity on gametocytemia was observed at the time of vector feeding, most likely because infected lizards were processed during various points in the course of the infection. The proportion of sandflies infected and oocyst burden were positively associated with higher gametocytemia. This has been a consistent finding in *Plasmodium* transmission studies on species infecting both human and non-human vertebrate hosts, including *P. mexicanum* (Taylor et al., 1997a,b; Taylor and Read, 1998; Schall, 2000). Transmission success was influenced by vector species. The more abundant sandfly, *L. vexator* (Table 3), is a more competent vector, carrying more oocysts than *L. stewarti*.

Although there was no evidence of an effect of clonal diversity on transmission success, there was a hint of a specific genotype effect, as detected by significant replicate effects within treatments. For example, allele "H" at locus Pmx306 was found in two groups, and both of those produced lower gametocytemia and oocysts burdens. Overall in the parasite metapopulation, this allele is relatively common compared with others at that marker, with a frequency of ~17% (data not shown; Vardo and Schall, 2007); therefore, it is perplexing that an allele that may have reduced transmission success remains common among hosts at the study site.

The similarity in oocyst burden between mixed and single-clone infections was not due to a substantial genetic bottleneck during transmission. All clones were passed on to the sandfly vector and survived to the oocysts stage in 74% of the sandfly feeds examined. The microsatellites used in this study have been shown to amplify minority clones within infections that are 10× less abundant than the predominant genotype (Vardo-Zalik et al., 2009). This finding implies that the failure to amplify some alleles present in the lizard at the time of feeding, but not in the oocyst population, was most likely due to absence rather than non-amplification. When genotyping microsatellite markers from a blood sample, DNA from both meronts and gametocytes is combined. Therefore, the results indicate that all clones in an infection typically produce mature gametocytes capable of successfully mating in the vector concurrently. The finding of similar oocyst burdens between the two groups suggests that the overall transmission potential of each clone within a multi-clonal infection is lower than that for a genotype in their single-clone counterparts.

A study on the transmission potential of multiple *P. falciparum* genotypes (gauged by the surface antigen markers MSP1 and MSP2) also found that the majority of clones within an infection were transmitted to the mosquito vector, although their relative proportions were not assessed (Huber et al., 1998). The simultaneous production of gametocytes by each genotype within mixed-clone infections may be a common case for malaria parasites, providing the opportunity for out-crossing, a scenario still debated (Anderson et al., 2000; Razakandrainibe et al., 2005; Annan et al., 2007; Mzilahowa et al., 2007). The occurrence of random mating between genotypes would thus allow for an increase of genetic diversity, via recombination, rather than a reduction during the transmission cycle.

The results presented here on the role of clonal diversity on the transmission biology of *P. mexicanum* differ from those reported for the rodent malaria parasite *P. chabaudi* studied in laboratory mice. Clonal diversity is not associated with variation in transmission

success for *P. mexicanum*, but in some *P. chabaudi* studies, mixed infections (harbouring two to three clones) produce more gametocytes than single-clone infections and, in some cases, are more infectious to mosquito vectors and produce more oocysts than single-clone infections (Taylor et al., 1997a,b; Taylor and Read, 1998). Other studies utilising the *P. chabaudi* system, however, have shown that the number of clones itself is not the key player in determining transmission success, but that it is the competitive ability of the individual clones within an infection (de Roode et al., 2005b). The parasite clones utilised in those experiments each express different virulence and growth phenotypes, something I have not been able to address for *P. mexicanum*. However, the hint that one allele at marker Pmx306 is associated with lower gametocyte levels does suggest the presence of genetic diversity in the parasite population for an important life history trait.

The relative proportion of the microsatellite marker alleles in the lizard matched those in the vector for *P. mexicanum* more frequently than expected by chance. For the two-clone infections, 89% of cases matched exactly, while 37% matched in infections with three to four genotypes (Fig. 2c). These results illustrate that not only are all genotypes within the lizard producing mature gametocytes at the same time, but they do so in the same proportions as their overall density in the lizard (measured by meronts and gametocytes). Therefore, it does not appear that clones within mixed infections out-compete each other for transmission opportunities. Studies on *P. chabaudi* have provided confounding results in terms of the relative proportion of genotypes in the vector and mouse hosts (Taylor et al., 1997a,b; de Roode et al., 2005b). These differences argue that cross-species comparisons (and indeed, comparisons within species at different geographic sites) will be productive for an understanding of the transmission biology of malaria parasites.

The transmission biology of malaria parasites could result in a loss of genetic diversity overall in the parasite metapopulation, and a reduction in the number of mixed-clone infections, in two ways.

Firstly, each transmission event could result in bottlenecks for clones as they transit from one host to the other, become established and reproduce. Second, existing infections appear to restrict entry of novel clones for *P. falciparum* and *P. mexicanum*, a process termed premunition (Smith et al., 1999; de Roode et al., 2005a; Vardo et al., 2007). Substantial bottlenecks do not appear to occur for the portion of the *P. mexicanum* life cycle from lizard to sandfly studied here because most clones become established, including those that were in relatively low proportions in the lizard's blood. A similar pattern appears to be true for *P. falciparum* (Huber et al., 1998).

The overall high genetic diversity observed for *P. mexicanum*, with mixed-clone infections very common at sites where transmission intensity is low (Vardo and Schall, 2007), argues that there is no important genetic bottleneck driven by the failure of clones to become established in the vector. Further studies that examine the possible loss of genetic diversity from the oocyst to sporozoite stage and subsequent infection into a new host are warranted.

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