Clonal Diversity of the Malaria Parasite Plasmodium Mexicanum: Diversity Over Time and Space, and Effects on the Parasite’s Transmission, Infection Dynamics and Virulence

Anne Vardo-Zalik
University of Vermont

Follow this and additional works at: https://scholarworks.uvm.edu/graddis

Recommended Citation

This Dissertation is brought to you for free and open access by the Dissertations and Theses at UVM ScholarWorks. It has been accepted for inclusion in Graduate College Dissertations and Theses by an authorized administrator of UVM ScholarWorks. For more information, please contact scholarworks@uvm.edu.
CLONAL DIVERSITY OF THE MALARIA PARASITE *PLASMODIUM MEXICANUM*: DIVERSITY OVER TIME AND SPACE, AND EFFECTS ON THE PARASITE’S TRANSMISSION, INFECTION DYNAMICS AND VIRULENCE

A Dissertation Presented
by
Anne M. Vardo-Zalik
to
The Faculty of the Graduate College
of
The University of Vermont

In Partial Fulfillment of the Requirements For the Degree of Doctor of Philosophy Specializing in Biology

October, 2008
Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, specializing in Biology.

Dissertation Examination Committee:

Joseph J. Schall, Ph.D.
C. William Kilpatrick, Ph.D.
Lori Stevens, Ph.D.
J. Ellen Marsden, Ph.D.
Frances E. Carr, Ph.D.
Abstract

The biology of malaria parasites, *Plasmodium spp.*, may be influenced by the presence of genetically distinct conspecific clones within a single infection, resulting in competition for host resources and transmission, and increased virulence for the vertebrate host. The extent of within host diversity, however, may be limited because overall clonal diversity could be reduced by the transmission biology of *Plasmodium* and variation in local prevalence. I examined clonal diversity of a natural malaria parasite-host association, *P. mexicanum* in its hosts, the western fence lizard, *Sceloporus occidentalis*, and sandflies, *Lutzomyia vexator* and *L. stewarti*, at a site in California ("Hopland"). Using microsatellite markers I characterized for the parasite, I examined (i) diversity within and among infections over time and space, (ii) transmission success of clones into both vector and lizard, (iii) the effects of clonal diversity on the parasite's infection dynamics and virulence for the lizard.

From 1996 to 2006, clonal diversity varied both temporally and spatially, with slightly more multiclonal infections detected during years of high vs. low parasite prevalence (88% vs. 78% for sites with the highest prevalence at Hopland). Spatially, low prevalence sites (< 1% of lizards infected) had fewer multiclone infections (50%). Thus, even when prevalence drops over time, or at sites with chronically low prevalence, clonal diversity of the parasite remains high.

Using natural and induced infections in the lizard, I found that multiclonal infections are no more infectious to vectors than single-clone infections, and almost all clones transfer successfully when the insect takes a blood meal. A competition experiment demonstrated that infections block new genotypes from entering a lizard host. Thus, multiclonal infections are likely to be established when vectors feed on a complex infection and transmit those parasite clones to an uninfected lizard. The transmission biology of *Plasmodium* thus allows for the maintenance of genetic diversity in the parasite population.

Finally, I examined the effects of multiclonality on the parasite's infection dynamics and virulence to the lizard host. Induced infections harboring a single or multiple clones had similar overall growth rates and maximal parasitemia, but multiclonal infections had significantly higher investment in gametocytes, suggesting competition for transmission. In addition, variation in parasite growth and density was greater for multiclonal infections, with approximately 1/3 displaying high replication rates and final parasitemia. Virulence measures indicated that weight change and proportion of immature erythrocytes was consistent for infections with 1, 2, 3 or > 3 clones, but the highly diverse infections had greater blood hemoglobin and glucose and more rapid clotting rates. Compared with the noninfected control lizards, highly diverse infections (3+) had higher blood glucose levels but similar hemoglobin levels.

I have found that genetic diversity of the malaria parasite *Plasmodium mexicanum* varies both temporally and spatially, although overall diversity remains high. The transmission dynamics of the parasite maintains high genetic diversity within infections. Additionally, diversity within hosts plays a significant role in variation of infection dynamics and virulence.
Citations

Material from this dissertation has been published in the following form:


Vardo, AM and Schall JJ. (2007). Clonal diversity of a lizard malaria parasite, Plasmodium mexicanum, in its vertebrate host: role of variation in transmission intensity over time and space. Molecular Ecology, 16: 2712-2720

AND

Material from this dissertation Appendix A has been published in the following form:


AND

Material for this dissertation has been accepted for publication to Ecology on March 25, 2008 in the following form:

Vardo-Zalik, AM and Schall, JJ. Clonal diversity alters the infection dynamics of a malaria parasite (Plasmodium mexicanum) within its vertebrate host.

AND

Material for this dissertation has been submitted for publication to Parasitology on February 11, 2008 in the following form:

Vardo-Zalik, AM and Schall, JJ. Clonal diversity within infections and the virulence of a malaria parasite, Plasmodium mexicanum.
Acknowledgements

I am grateful to so many people for their help in the completion of my degree. Firstly, I would like to thank my advisor, Jos. J. Schall for inspiring me, believing in me when I felt no one would, and for being a continuous source of enthusiasm for our little critters. I only hope to be as charismatic as he when I have been teaching for 20 years.

I would like to thank my wonderful committee members, Bill Kilpatrick, Lori Stevens and Ellen Marsden for their support, encouragement and constructive feedback. I have learned so much from each of them and I am grateful to have worked with them.

The Hopland Research and Extension Center was my home away from home away from home for many summers and I could not have felt more welcome. Bob Timm and Bob Keiffer were incredibly accommodating to my research needs. Chuck Vaughn was a great lab host and I am very thankful to him for giving up half of his lab every summer- I apologize for any runaway lizards. Jane and Amber were smiling faces to greet me everyday and were always there to help with housing needs and office emergencies. I would like to thank Bill Marston and Jonas Dumaine for their support and generosity with field vehicles and various housing maintenance problems (including being locked out of both the bunkhouse and trailer).

The Biology Department of the University of Vermont has been such supportive place during my many years here. Wendy, Jen and Linda were always there and quick to answer any question I had, no matter how trivial. The Ecology and Evolution faculty have been great sounding boards for my research and I appreciate all their amazing
feedback during my ecolunch presentations. I especially thank Nick Gotelli and Charles Goodnight for help with my analyses.

The EE graduate students have been remarkable during my time here. I think that the only people who know what you are going through as a graduate student are those in the same shoes as yourself. Steve Hudman, Sarah Whitman, Laura Hill, and Kate Farrell were amazing colleagues and I enjoyed my time working with them and listening to their presentations. My lab mate Ellen Martinsen has been a tremendous ‘spirit’ in our lab. She always knew when to give a quick ‘pick me up’ when things got crazy. I will miss her energy and her companionship. Ryan Norris has been a friend, mentor and valuable colleague and I could not have gotten this far without his constant source of support (and his constant supply of Dr. Pepper). I will never forget “Them Beans”, our weekly mammal quizzes in the fall and the rodent skull necklace. Finally, Amy (Wakefield) Norris has been a truly remarkable friend and researcher. I will miss our weekly ‘teas’, her help in the field and her smiling, red face.

To my amazing undergraduates, especially my BIO 246 labs, thank you. Teaching has always been my passion and it was a wonderful experience sharing that with them. My undergraduate researchers throughout the years have instilled in me a deeper understanding of mentoring and teaching. Jennifer Fricke, Alice Ford, Julie Dao, Dan Wheeler, Kim Kaufhold, Ben Blumberg and Holly Archer have been the hardest working undergraduates I know. I have enjoyed working closely with each of them and wish them the best of luck in their future careers.
I am grateful to the funding agencies, including NSF, Vermont Genetic Network, HELIX and APLE, for financing my dissertation studies and my undergraduate researcher projects. Their funding allowed me to expand my dissertation research and explore clonal diversity of *Plasmodium mexicanum* in all aspects.

Finally, I could not have achieved anything in my life without the wonderful love and support of my family. Chris, Kristine, Joy, Jonathan and Michael-Man have been so amazing throughout my journey here. I cannot put into words how lucky I feel to have them. My parents, Jackie and Chuck, have been there for me through some of the most trying times, and I only hope that my support and love for them matches theirs for me. I dedicate this dissertation to them. Mom and Dad Z, Aaron, and Denise are the best family-in-law I could have asked for. Their encouragement from day one has been insurmountable and I will be forever grateful.

Last, but certainly not least, my husband Nate. Words cannot describe my gratefulness for all his love, confidence, support, advice and good cooking. I enjoyed being his boss for a summer and am grateful for the amazing fieldwork he accomplished. Most of all, I thank him for being my rock and never letting me quit what I love. Thank you from the bottom of my heart.
# Table of Contents

Citation ........................................................................................................................................................................... ii

Acknowledgements .................................................................................................................................................................. iii

List of Tables ........................................................................................................................................................................ xi

List of Figures ......................................................................................................................................................................... xiii

Chapter

  1. Comprehensive Literature Review ................................................................................................................................. 1

  2. Calibration of Microsatellite Methods ............................................................................................................................. 15

      Abstract ............................................................................................................................................................................. 15

      Introduction .................................................................................................................................................................... 15

      Methods and Results ......................................................................................................................................................... 16

      Test 1: Detecting Mixed-clone infections ..................................................................................................................... 16

          1.1 Test 1a ................................................................................................................................................................. 16

          1.2 Test 1b ................................................................................................................................................................. 18

          1.3 Test 2 ................................................................................................................................................................. 18

          1.4 Test 3 ................................................................................................................................................................. 20

          1.5 Test 4 ................................................................................................................................................................. 21

          1.6 Summary ............................................................................................................................................................ 22

      Test 2: Genotypes over time ............................................................................................................................................ 24

          2.1 Test 1 ................................................................................................................................................................. 24

          2.2 Test 2 ................................................................................................................................................................. 25
3. PCR detection of lizard malaria parasites: prevalence of Plasmodium infections with low-level parasitemia differs by site and season

Abstract

Introduction

Methods

Results and Discussion

Acknowledgments

Literature Cited

4. Clonal diversity of a lizard malaria parasite, *Plasmodium mexicanum*, in its vertebrate host: role of variation in transmission intensity over time and space

Abstract

Introduction

Methods

Study areas

Molecular Methods

Measures of Clonal Diversity
Results .................................................................................................................. 73
Clonal Diversity Within Infections ........................................................................ 73
Overall Clonal Diversity ....................................................................................... 73
Discussion ........................................................................................................... 75
Acknowledgments ............................................................................................... 81
Literature Cited ..................................................................................................... 82

5. Experimental test for premunition in a lizard malaria parasite

(Plasmodium mexicanum) ......................................................................................... 92

Abstract ................................................................................................................ 93
Introduction .......................................................................................................... 93
Methods ................................................................................................................ 95
Results .................................................................................................................. 97
Discussion ............................................................................................................ 99
Acknowledgments ............................................................................................... 101
Literature Cited ..................................................................................................... 102

6. Clonal diversity of a malaria parasite, Plasmodium mexicanum, and its transmission
success from its vertebrate to insect host ............................................................. 106

Abstract ................................................................................................................ 106
Introduction .......................................................................................................... 107
Methods ................................................................................................................ 109
Results .................................................................................................................. 114
Discussion ............................................................................................................ 116
7. Clonal diversity alters the infection dynamics of a malaria parasite

(Plasmodium mexicanum) within its vertebrate host

Abstract

Introduction

Methods

Study sites and collection of lizards

Genotyping infections

Experimental infections

Infection traits

Analysis

Results

Growth rates, maximal parasitemia, and first production of gametocytes

Variation in traits

Discussion

Acknowledgments

Literature Cited

8. Clonal diversity within infections and the virulence of a malaria parasite,

Plasmodium mexicanum

Abstract
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>164</td>
</tr>
<tr>
<td>Methods</td>
<td>166</td>
</tr>
<tr>
<td>Results</td>
<td>171</td>
</tr>
<tr>
<td>Discussion</td>
<td>173</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>179</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>180</td>
</tr>
<tr>
<td>Appendixes</td>
<td></td>
</tr>
<tr>
<td>A. Identification of microsatellite markers in <em>Plasmodium mexicanum,</em>\n</td>
<td>a lizard malaria parasite that infects nucleated erythrocytes</td>
</tr>
<tr>
<td>B. General Laboratory Methods, Protocols and Recipes</td>
<td>196</td>
</tr>
<tr>
<td>Comprehensive Literature Cited</td>
<td>203</td>
</tr>
</tbody>
</table>
List of Tables

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Donor lizard DNA extractions for first microsatellite calibration runs</td>
<td>31</td>
</tr>
<tr>
<td>2.2</td>
<td>Calibrations test 1a: DNA extractions used to create ranges of</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>multiclonal infections.</td>
<td></td>
</tr>
<tr>
<td>2.3</td>
<td>Calibration test 2: multiclonal infections across three loci.</td>
<td>33</td>
</tr>
<tr>
<td>2.4a</td>
<td>Calibration test 3: Experimental setup for detections of alleles by size</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>and concentration.</td>
<td></td>
</tr>
<tr>
<td>2.4b</td>
<td>Summary of Calibration test 3</td>
<td>35</td>
</tr>
<tr>
<td>2.5a</td>
<td>Calibration test 4: Allele detection with varying concentrations of DNA</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>for 3 microsatellite loci.</td>
<td></td>
</tr>
<tr>
<td>2.5b</td>
<td>Calibration test 4: Combinations and dilutions used for each of 10 runs</td>
<td>36</td>
</tr>
<tr>
<td>2.5c</td>
<td>Summary of results for Calibration test 4</td>
<td>37</td>
</tr>
<tr>
<td>4.1</td>
<td>Genetic diversity within natural infections of <em>Plasmodium mexicanum</em></td>
<td>86</td>
</tr>
<tr>
<td>4.2</td>
<td>Diversity measures for <em>Plasmodium mexicanum</em> over time and space</td>
<td>88</td>
</tr>
<tr>
<td>4.3</td>
<td>Alleles present during high prevalent years but lost during years with</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>low malaria prevalence</td>
<td></td>
</tr>
<tr>
<td>4.4</td>
<td>Heterozygosity excess for 3 <em>Plasmodium mexicanum</em> microsatellite loci</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>estimated for two different mutation models</td>
<td></td>
</tr>
<tr>
<td>6.1</td>
<td>Donor genotypes for transmission experiment</td>
<td>125</td>
</tr>
<tr>
<td>6.2</td>
<td>Transmission treatment groups and replicates</td>
<td>126</td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>6.3</td>
<td>Proportion of sandflies infected for each treatment</td>
<td>126</td>
</tr>
<tr>
<td>6.4</td>
<td>Summary data for each treatment (gametocytemia and oocyst burden)</td>
<td>127</td>
</tr>
<tr>
<td>7.1</td>
<td>Donor infections and genotypes for experimental infections</td>
<td>156</td>
</tr>
<tr>
<td>7.2</td>
<td>Donors, treatment groups and replicates</td>
<td>157</td>
</tr>
<tr>
<td>7.3</td>
<td>Summary data for infection traits</td>
<td>158</td>
</tr>
<tr>
<td>7.4</td>
<td>ANOVA results</td>
<td>160</td>
</tr>
<tr>
<td>8.1</td>
<td>Donor infections for virulence treatments</td>
<td>186</td>
</tr>
<tr>
<td>8.2</td>
<td>Summary data for 5 virulence measures</td>
<td>187</td>
</tr>
<tr>
<td>A1</td>
<td>PCR and primer data for 7 microsatellite loci</td>
<td>194</td>
</tr>
</tbody>
</table>
List of Figures

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Calibration test 1: Detection of 7 clones.</td>
<td>40</td>
</tr>
<tr>
<td>2.2</td>
<td>Calibration test 1b: Detection of multiple peaks.</td>
<td>40</td>
</tr>
<tr>
<td>2.3</td>
<td>Calibration test 2: multiple allele detection for 3 markers.</td>
<td>41</td>
</tr>
<tr>
<td>2.4</td>
<td>Calibration test 3: concentration and size biases.</td>
<td>42</td>
</tr>
<tr>
<td>2.5</td>
<td>Calibration test: genotypes over time</td>
<td>43</td>
</tr>
<tr>
<td>2.6</td>
<td>Calibration test: Repeatability</td>
<td>45</td>
</tr>
<tr>
<td>3.1</td>
<td>Sensitivity of nested PCR protocol in detecting weak infections.</td>
<td>63</td>
</tr>
<tr>
<td>4.1</td>
<td>Clonal diversity of <em>Plasmodium mexicanum</em> compared with <em>Plasmodium falciparum</em></td>
<td>92</td>
</tr>
<tr>
<td>5.1</td>
<td>Transmission success of multiple genotypes when inoculated into noninfected lizards.</td>
<td>105</td>
</tr>
<tr>
<td>6.1</td>
<td>Oocyst burden for sandflies feeding on infections with 1-3+ clones</td>
<td>129</td>
</tr>
<tr>
<td>6.2</td>
<td>Proportion of genotypes successfully transmitted to sandfly (1 or 2 loci)</td>
<td>130</td>
</tr>
<tr>
<td>7.1</td>
<td>Infection dynamics for infections harboring 1 or many genotypes.</td>
<td>162</td>
</tr>
</tbody>
</table>
Chapter 1
Comprehensive Literature Review

For my dissertation, I performed three major sets of experiments, each addressing a different aspect of diversity in *Plasmodium mexicanum*, primarily ‘clonal diversity’. Clonal diversity of a malaria parasite refers to the presence of more than one genetically distinct clone of the parasite within a single infection. I wanted to study diversity of *Plasmodium mexicanum* because of the implications it can have on multiple aspects of the parasite’s biology. For example, competition for resources within a single host could lead cohabitating genotypes to alter their reproductive cycles to produce more parasites, thereby hoarding host resources and transmission opportunities (Babiker *et al.* 1999; de Roode *et al.* 2003, 2005a; Eisen and Schall 2000; Read and Taylor 2001). In turn, this could increase the pathology to the host if the infection cannot be controlled (Bull 1995; de Roode *et al.* 2005b; Ewald 2004; Frank 1996; Mackinnon *et al.* 2002; Read and Taylor 2001; Schall 2002; Taylor *et al.* 1998; Vanbaalen and Sabelis 1995).

Additionally, the maintenance of diversity in a malaria parasite population is intriguing. From the moment I was introduced to this system, I was filled with questions- the main one being “How”? With such a small blood meal taken by the vector from an infected host, how can infections with >1 genotype be common? Is the same host bitten by multiple infected vectors each carrying a unique genotype of the parasite? How much diversity is lost during each transmission cycle? How can such a ‘small’ parasite be capable of detecting conspecifics and altering its course of infection?
To answer these questions, I would need to be able to do one major thing: detect diversity. Most molecular markers developed for malaria parasites are surface antigen proteins, which, although they can be useful and informative, are not neutral to selection and thus offer a biased view of diversity (Anderson et al. 1999, 2000). Before I started my PhD, the first microsatellite markers were developed for Plasmodium falciparum, and since then, markers for P. vivax and P. malariae have also been developed (Anderson et al. 1999, 2000; Bruce et al. 2007; Imwong et al. 2006). Microsatellite markers are useful in population genetic studies because they can identify individuals and are supposedly selectively neutral. These markers would allow me to address the questions that I wanted to answer, but were not available for P. mexicanum at the start of my dissertation. Nine months after starting my PhD, Joe Schall and I developed our first microsatellite marker, and 9 months later, we had characterized an additional six markers.

The first set of experiments in this dissertation describes the different methods I used to assess the usefulness of the newly developed microsatellite markers for Plasmodium mexicanum (Schall and Vardo 2007) and the sensitivity of our cytochrome b polymerase chain reaction (PCR) in detecting low-level infections (Vardo et al. 2005).

In Chapter 2, I describe the methods I used to examine the efficacy of microsatellite markers in determining diversity within and among infections of Plasmodium mexicanum. Through a series of different calibration tests, I mixed extracted DNA from infected lizards with known clonalities to determine detectability of multiple alleles, detection of rare clones, and repeatability for 1-3 of the most polymorphic markers. I also followed a series of infections overtime to examine changes in the parasite
population throughout the course of an infection. *Plasmodium* parasites in their vertebrate host are haploid, therefore each allele at a microsatellite marker represents a single genotype. (1) I have found that microsatellites are useful markers in examining diversity of *Plasmodium mexicanum* and allow for the detection of up to 6 different alleles per marker. I suggest using a standardized ‘cutoff’ for allele peak strength when reading the pherograms because this is a very subjective process. Any peak below 50 fluorescent units is not taken into account in my studies. (2) “Rare” alleles within an infection can be detected as long as their concentration in the blood is at least 1/10 the concentration of the major genotype. Utilizing multiple microsatellite markers will allow for a more sensitive detection of rare clones. (3) In addition, samples from the same infected individual should be examined over time, as clones appear to wax and wane in their abundance over the course of the infection and extremely rare clones may become the major clone within a few days time. (4) Allele detection can be hampered by stutter patterns. These stutter peaks are often very small compared to the major, true allele, but because ‘real’ alleles can fall in these regions, detection of clones within stutter areas can be subjective. I have found that detecting ‘true’ alleles in post-stutter peaks is rather simple, as the post-stutter peak (with the real allele) is higher than any pre-stutter peak. Detecting alleles falling in pre-stutter regions, however, is more difficult and I suggest using a 50-75% cut off rule when determining if a stutter peak before the major allele is real or not.

In Chapter 3, I present a paper published with an undergraduate researcher and my advisor that examines the sensitivity of polymerase chain reaction, PCR, in detecting
Plasmodium infections (Vardo et al. 2005). This protocol was used to select noninfected fence lizards (Sceloporus occidentalis) for my experiments so I wanted to ensure I could accurately detect infectivity. In the Caribbean, many infections are not detected by simple microscopic investigation and are therefore considered “negative”. However, using a sensitive PCR protocol, we found that infection rates are higher than suggested by microscopy alone (termed “false negatives”), and detection of infection by microscopy differs depending upon season. For the temperate California system, “false negatives” are extremely rare. In addition, we found that our PCR protocol can detect infections with as little as 1 parasite cell per millions of red blood cells, rendering this technique a practical method for ensuring the noninfected nature of lizards.

The second set of experiments for my dissertation focuses on the extent of genetic diversity both within and among infections of Plasmodium mexicanum. A preliminary survey during the calibration methods (Chapter 2) suggested that diversity is high and I wanted to examine how diversity may change depending upon site and year, how diverse infections become established (do existing infections prevent superinfection?), and how the transmission biology of Plasmodium mexicanum influences diversity within infections.

Chapter 4 presents a study on the diversity of Plasmodium mexicanum both within and among infections and how that diversity is altered depending upon site (high or low prevalence) and year (high or low overall prevalence) (Vardo and Schall 2007). Two hundred forty-six natural infections captured between 1996 and 2005 were analyzed at 6 microsatellite markers and data were sorted by year (high years 1996-1998, low years
2001-2004 and lowest year 2005) and by site (low prevalence 1-2% and high prevalence > 10%). We calculated the proportion of multiclonal infections, diversity at each microsatellite marker (H), and mean number of alleles per marker to examine how diversity changes over time. The proportion of multiclonal infections and diversity (H) at each marker was similar across years, even during the lowest prevalence year when only 6% of lizards was infected. The prevalence at each site, however, affected the clonality of infections, with low prevalence sites having fewer multiclonal infections, yet overall diversity at each marker was still high. After 5 years of consistent low prevalence (2001-2005) a drop in the mean number of alleles at each marker was noticed. The majority of the lost alleles were rare during years when they were detected. These data are comparable to Plasmodium falciparum, with higher diversity and multiclonality found in areas of higher transmission (higher prevalence). The likelihood that the parasite populations had experienced a genetic bottleneck was examined under two different microsatellite mutational models and we found that heterozygosity was in excess under only one of the models (IAM, not SMM). The results from this study suggests that even though alleles can be lost after years of low transmission, overall diversity remains high.

Chapter 5 details an experiment I performed with an undergraduate researcher, Kim Kaufhold, examining the competition for establishment of novel clones within already infected hosts (Vardo et al. 2007). This experiment had two major objectives: to determine the presence of within-host competition and to investigate the likelihood of multiclonal infections being initiated from a single infectious vector bite. We found that, although 100% of infections establish within noninfected hosts, only 36% of novel clones
established within already infected hosts. Infections that successfully ‘eliminated’ novel incoming clones from establishing were ‘older’ in that they had a higher gametocyte parasitemia than infections that succumbed to invasion. These results suggest that within-host competition exists, and that clones can detect the presence of conspecifics. Whether the inability for novel clones to establish was due to parasite-mediated or host responses is unclear. From this study, we propose that multiclonal infections are more likely initiated via the bite from a single infected vector harboring > 1 genotype because older infections prevent superinfection and the chances for repeated bites from different infectious vectors within a short period of time seems unlikely in this system, where vectors are commonly in low abundance. Our findings are comparable to the competitive interactions between novel and established clones detected in *Plasmodium falciparum* and *P. chabaudi* infections (Smith *et al.* 1999; de Roode *et al.* 2004).

Finally, in Chapter 6, I examine the transmission efficiency of mixed-clone infections and how the transmission biology of *Plasmodium mexicanum* could lead to a bottleneck in genetic diversity. In Chapters 3 and 4, I found that diversity within and among infections is high at the Hopland field site in Northern California, which is intriguing because of the transmission dynamics of the parasite. I utilized induced experimental infections with 1, 2 or 3+ clones to test whether there is a reduction in the number of genotypes successfully transmitted to the sandfly vector and whether mixed-clone infections are more or less ‘infectious’ to the vector. Multiclonal infections are proposed to be either more or less infectious because of within-host competition for transmission opportunities; with every clone fighting for each transmission chance, it seems plausible
that the more clones within an infection, the more transmission stages and hence, the
greater efficiency. (I will expand more upon infection dynamics and parasite
productivity in Chapter 7.) Wild caught sandflies (*Lutzomyia vexator* and *L. stewarti*)
were allowed to feed on the experimental infections and after 5 days, were dissected to
assess infectivity and oocyst burden (Fialho and Schall 1995). Neither infectivity nor
oocyst burden were correlated with clonality within infection, rather, infection
gametocytemia, not related here to clonality, was the driving force for both these
variables. Genotyping a random sample of infected midguts for 2 microsatellite markers
revealed no significant reduction in diversity during transmission. These findings
indicate that a mixed-clone infection can be initiated by the bite of a single infected
vector. Additionally, single-clone infections are no less infectious or successful in
transmission as their multiclonal counterparts- an opposite finding than that from a rodent
malaria model (*Plasmodium chabaudi*) (Taylor *et al.* 1997).

The final set of experiments for my dissertation focuses on the effects of genetic
diversity within infections both for the parasite and the host. Previously, Eisen and Schall
noted that infections of *Plasmodium mexicanum* vary greatly in their life history traits
(termed herein as infection dynamics) (Eisen and Schall 2000). This variation among
infections could be due to complexity of infections. Interaction between clones within a
single infection could lead to variety of outcomes, including competition for host
resources and transmission opportunities, which could greatly alter the parasitemia of a
given infection (Read and Taylor 2001). Similarly, the presence of multiple clones could
alter the pathology to the host by multiple pathways, such as eliciting a stronger immune
response (costly to the host) and using up greater amounts of host resources (Anderson and May 1979; Bull 1995; Ewald 2004; Read and Taylor 2001). In the following experiments, I aimed to study the effects within-host competition has on the parasite’s infection dynamics and whether or not multiclonal infections are more virulent to the lizard host.

In Chapter 7, I present an experiment addressing how parasite infection dynamics are influenced by within-host competition (Vardo-Zalik and Schall 2008). Using experimentally infected lizards with known ranges of parasite genotypes (1 to >3), I followed 69 infections over 80 days and examined changes in parasite growth rates (both asexual and sexual transmission stages), in maximal parasitemia for each parasite stage and date of first patency in the blood. Grouping infections by donor within clonal treatments, I found that simple infections had a limited range of infection dynamics; that is, all infections within this treatment, regardless of donor, produced similar numbers of parasites and at the same rate. Multiclonal infections (>1 clone) however, produced variable traits, with more than 1/3 of infections growing faster and/or producing more parasites of one stage or another while the other infections produced similar results as the simple infections. No genotype effect was observed, for the same genotypes that were common in the 'outlier' infections were also common in single-clone infections and multiclonal infections registering in the ‘single-clone’ range. Additionally, infections that were ‘outliers’ for one trait were not always ‘outliers’ for other traits. It appears that a host effect, such as immune competency, may be present. Finally, infections harboring three or more genetic clones produced higher gametocyte parasitemias at a faster rate.
than their 1 and 2-clone counterparts. Timing of the production of first gametocytes was consistent across all treatments. These results illustrate that within-host competition does alter the infection dynamics in diverse infections, most notably by increasing the production of transmission stages, suggestive of clonal competition for transmission opportunities.

Chapter 8 presents my final dissertation experiment on clonal diversity of *Plasmodium mexicanum* focusing on the virulence of simple and diverse infections. Virulence refers to the pathology experienced by the host because of the parasitic infection. I use the term here loosely to imply any pathology, whether host or parasite mediated. *Plasmodium mexicanum* is a virulent saurian malaria parasite. *Sceloporus occidentalis* infected with *P. mexicanum* suffer a wide range of afflictions including reduced hemoglobin and glucose concentrations, reduced stamina, the inability for males to hold territories and an overall reduction in fitness (reduced clutch size for females and testes size for males) (Dunlap and Schall 1995; Schall *et al.* 1982; Schall 1983, 1990a, 1990b, 2002). Using the same experimental lizards as my previous infection dynamics study, I measured weight loss, proportion of immature erythrocytes, blood hemoglobin and glucose levels, mortality and blood coagulation. All measurements except for weight loss were taken at the end of the experiment. Clonality within infections had no effect on weight loss (all lizards gained weight, but there was not a difference between treatments), proportion of immature erythrocytes in circulation, and mortality. Diverse infections (3 or more clones) however, had higher blood glucose levels than both the noninfected controls and the simpler infections. In fact, the level of glucose found in diverse infections was similar to that of
newly caught noninfected lizards from the field, suggesting that the stress of captivity may have resulted in the lower blood glucose observed for our captive controls. Diverse infections also had higher blood hemoglobin when compared with infections harboring 1 or 2-clones, but similar levels to the control group. Again, it appears that multiclonal infections pose less harm to their lizard host. Finally, blood from infected lizards has the tendency to clot rapidly after leaving the host, even in heparinized capillary tubes. I found that diverse infections (>3 clones) have a higher chance of blood clotting (up to 86% lizards clot) than noninfected lizards and lizards with 1-3 clones. The exact mechanism of this clotting is unknown- it could be mediated by the parasite to enhance transmission, to cluster noninfected cells to infected cells for easier infection, or the clotting could be a by product of the higher blood glucose in these infections. I am also unaware as to the ‘benefit’ of having blood rapidly clot for a lizard- it could be helpful after injury, but harmful depending upon whether or not clots form within circulation.

Parasitemia did not have an effect on any of the virulence measures. In summary, clonal diversity does play a role in altering the virulence of Plasmodium mexicanum infections, but the outcome appears to oppose theory; multiclonal infections in P. mexicanum are less virulent, and possibly even beneficial, to their host when compared to 1 and 2-clone infections.

My dissertation has explored the extent of genetic diversity within Plasmodium mexicanum populations and how that diversity affects multiple aspects of the parasite’s biology. As with any complex system, I have come away with more questions and experimental ideas than answers, but I am told that is how research should progress.
Microsatellite markers are a useful tool for detecting genetic diversity and but I propose further research into the calibration of methods to reduce the amount of subjectivity currently needed when analyzing these data.

Future studies on clonal diversity within *Plasmodium mexicanum* infections should examine host genotypes and the immune response of each infected lizard to a variety of clonalities and clonal pairings, and also parasite genotype abundance within infections (Mackinnon *et al.* 2002). The results I have found from the infection dynamics and virulence experiments suggests that there is more than just infection clonality affecting these factors.

Finally, a thorough vector survey of each high prevalence field site at Hopland will allow for a more accurate account of the transmission biology of *P. mexicanum*. Genetic diversity of the parasite is high at each field site, yet vectors are rare, or at least they appear to be. Although the basic transmission cycle of the parasite is understood, it may be more complex than we think. Other vectors may exist, which could explain the high prevalence of the parasite in areas where sandflies have never been captured (Schall and Smith 2005). Or, sandflies may prefer habitats other than the rodent burrows that have been surveyed in the past. Either way, the transmission dynamics of this system should be reexamined. Doing so will open the door to an array of experiments to examine the population genetics of *Plasmodium mexicanum*. 
Literature Cited


Chapter 2
Assessing clonal diversity of *Plasmodium mexicanum* using microsatellite markers: verification of techniques

Abstract

The sensitivity and usefulness of newly characterized *Plasmodium mexicanum* microsatellite markers used to detect within-host diversity were assessed. Extracted DNA from naturally infected lizards was mixed in differing proportions to detect ‘rare’ clones within an infection, the maximum number of alleles clearly detectable at a microsatellite marker, and repeatability of results. The detection of up to six individual alleles per marker is possible, but alleles similar in size often cause an underestimate of diversity. No new alleles were observed in infections followed over a three-month period, although the ‘abundance’ of each genotype within the infection did change over-time. Thus, these markers are not mutating at a rate that would generate new genotypes of the parasite and overestimate diversity. These results render microsatellite markers a useful tool in assessing within and among-host genetic diversity of *P. mexicanum*.

Introduction

The calibration and verification of new molecular techniques that are used to explore a novel system is a critical initial goal of any new research effort. For a study on the clonal diversity of the malaria parasite *Plasmodium mexicanum* in lizard hosts, new molecular markers—microsatellites—were employed. Prior to this study, only a few investigators have used these microsatellites in studies of genetic diversity for the *Plasmodium of
humans, so ‘ready to go’ protocols that could cross-over for use with a lizard malaria parasite have not yet been perfected (Anderson et al. 1999, 2000). In fact, none of the published microsatellite primers for *Plasmodium falciparum* were successful in amplifying the DNA of a lizard malaria parasite, so new markers had to be developed. After development, it was necessary that these markers be tested to ensure that results were repeatable and that all clones present in an infection could be detected. In addition, a method for detecting the presence of alleles that differ in size by only one repeat (ATT repeat for all loci) was warranted, as microsatellite alleles often have a pre-allele, or stutter, peak which is 3bp less than the true allele. Any alleles falling in the stutter range may be discarded as ‘false’ when the allele is really present.

The following section describes the different tests used to verify the method’s ability to detect clones and to repeat results for one to three of the most polymorphic loci for this species.

**Methods and Results**

**Test 1: Detecting Clones**

1.1 Detecting mixed clone infections: Test 1

Infections of a malaria parasite commonly harbor more than one genetically distinct form of the parasite (clones) within a single infection. Detecting these clones, and assessing the number of clones within an infection is critical for any study on the biology of clonal diversity. That is, testing hypotheses requires an ‘accurate’ estimate of the number of parasite clones present in an infection. Being able to detect a multi-clonal infection, and to determine the maximum number of clones that could be distinguished,
was the first goal. One well-performing marker, Pmx306 (Schall & Vardo, 2007) was used for the first test. For this test and all other calibration tests described herein, DNA was extracted from dried blood dots stored on filter paper using the Qiagen DNeasy kit.

Extracted DNA from 6 different lizards found in a preliminary study harbored 1 – 2 clones each (alleles of the Pmx 306 locus) was used for this first test. Combining 5 µl of each DNA extraction in a 1.5 ml tube, the mix of DNA extraction products contained 7 different genotypes (Table 2.1). For the PCR, 2 µl of the extraction mixture was used as the template DNA. The product was diluted 1:20 H₂O for genotyping on an ABI sequencing instrument at the University of Vermont’s Cancer Research Facility. The data were examined with Genotyper 2.0 software (ABI). The resulting pherogram showed 6 peaks, with each peak indicating different clones (Figure 2.1). These were scored based on knowledge of the actual genotypes in the mixture of extracted DNA. However, two of the peaks were only 3 bp different in size, so it would have been difficult to determine if these were true alleles or stutter peaks without prior knowledge of the clonal makeup of the sample. Therefore, if an observer had no prior knowledge of the true clonal diversity within the sample, it would have been scored as having “4 or more” clones. In this experiment, the larger fragment sizes, in general, produced smaller peaks. This could be due to either those alleles being in lower concentration or by the competitive nature of PCR and the ABI instrument differentially detects smaller allele fragments.

1.2. Detecting mixed clone infections: Test 1b
Shortly after the first trial, the instrumentation at the local sequencing facility (Vermont Cancer Center) was upgraded to an ABI 310 and a new software package was available for use (GeneMapper 3.5 vs. Genotyper 2.0). A trial was therefore repeated to test the sensitivity of the new instrument and software. Four different combinations of known clones (from single or two-clone infections) were used (again mixing DNA extraction product). These combinations were: 2 clones (2 µl of each of infections from two lizards), 3 clones (1 µl from each of three lizards), 4 clones (1 µl from each of four lizards) and 5-6 clones (1 µl each of five lizards) (Table 2.2). Again, 2 µl from each mix was used as DNA template for PCR amplification of locus Pmx306. The product was diluted 1:10 H₂O for genotyping. For the 2, 3 and 4-clone mixtures, I was able to distinguish all alleles; however, some were much smaller than others (primarily allele size 185) (Figure 2.2). For the 5-6 clone mixture, I again saw 4 clear alleles, but two of the alleles added into the mixture fell in the stutter range of a previous allele (Figure 2.2).

1.3: Detecting mixed clone infections: Test 2

There were two problems with the two tests given above. 1. Alleles were used that could fall within the stutter range of other alleles in the mixture, resulting in a reduction in the number of alleles scored, and 2. the abundance of each clone within the infections differed from lizard to lizard. Although there is no clear way to measure the abundance of each clone within an infection, it can be estimated using the fluorescent units on the pherogram (Anderson et al. 1999, 2000; ABI Genescan manual). By combining DNA with differing proportions of clones, replication of the most abundant clones was likely
favored in the PCR. Therefore, this test was performed again, using two additional loci, and lizards that harbored clones with similar fluorescent unit readings on the pherograms (and thus, presumably similar parasite densities) (Table 2.3).

For Pmx306, 2 µl of DNA extraction product was combined from four different infected lizards. Six alleles were mixed, each with a size that should cause minimal stutter confusion (Table 2.3). For the PCR, 5 µl of this mixture was used as DNA template and the PCR thermalcycler program appropriate for the locus was used (Appendix A). The product was diluted 5 µl product: 90 µl H₂O for genotyping. From the resulting pherogram, 4 of the 6 alleles were clearly visible (Figure 2.3a).

For locus Pmx747, 2 µl of extracted DNA from 5 different lizards was mixed together, forming a 10µl DNA mixture with 5 different alleles (Table 2.3). Five µl of this was used as template DNA for the PCR reaction and the program suitable for the locus was used (Appendix A). The product was diluted 5 µl product: 90 µl H₂O for genotyping. The resulting pherogram showed 4 clear alleles (Figure 2.3b).

For a third locus, Pmx732, 2 µl of extracted DNA from 4 different lizards was mixed together, forming an 8 µl DNA mixture with 5 different alleles (Table 2.3). Five µl of this was used as template DNA for the PCR reaction and the thermalcycler program for Locus Pmx732 was used (Appendix A). The product was diluted 5 µl product: 45 µl H₂O for genotyping. The resulting pherogram showed only 2 clear alleles and a very weak signal (Figure 2.3c). This is a continuing problem with this locus. That is, Pmx732 tends to produce a weak signal, and most likely clones are missed. Pmx732 therefore could not be used as a single indicator of clonal diversity, but only when combined with data for
other loci. This was the general protocol eventually followed: to always use several loci for any study of clonal diversity.

1.4: Detecting rare clones: Test 3

PCR is a competitive process that favors the most common template in the reaction mixture. Therefore, if two clones are present, with one much more common than the other, it is possible that only the common clone will be detected on the pherogram. The following tests examined the outcome if two competing DNA templates (two alleles) differed in concentration. Again, multiple tests were completed using different loci and the two different ABI instruments.

For the first trial, Locus Pmx306 was used for the assays. Five alleles were chosen for mixing in various concentrations with lengths (in bp) 171, 181, 194, 197, and 209 (Table 2.4a). Note that some allele pairs are similar in length, whereas others are substantially different. Table 2.4b shows the different concentrations and combinations of extracted DNA used in the trial. The PCR products were diluted 1:10 H₂O for genotyping and were run on the new ABI machine with new software (some of the allele calls differ by 1-2 bp from those scored using the original instrument for the same lizard).

This trial demonstrated that smaller fragments (smaller alleles) and alleles with a higher concentration were more likely to be seen on the pherogram (Figure 2.4). However, in most cases, both alleles were seen. This result suggests that the “size” and “concentration” biases are, at least sometimes, not so great as to miss alleles.

1.5: Detecting rare clones: Test 4
The final tests performed to demonstrate the sensitivity of the PCR amplification utilized three loci (Pmx306, 747 and 732) and alleles with similar fluorescent units. For each locus, I chose three lizards with single clone infections harboring alleles fairly spaced apart in size. I tried to have two alleles similar in size and one of a different size, to emphasize the above results (Tables 2.5a-c). I tested 1:1, 1:10 and 1:5 combinations. The 1:1 combinations of alleles were made by putting 4 µl of each DNA extraction together; for a 1/10 dilution, 5 µl of DNA was mixed with 50 µl H₂O and for a 1/5 dilution, 5 µl DNA was mixed with 25 µl H₂O. For these PCRs, 2.5 µl of each mixture was pipetted directly into the PCR vial; a separate mixture vial for each combination was not made (unlike the above trials). If a band showed on the PCR product gel, the product was diluted 5 µl product: 90 µl H₂O for genotyping. If no band was produced, 5 µl of product was mixed with 45 µl H₂O.

This set of tests showed mixed results, depending upon which locus was examined (Table 2.5c). For locus 306, all tests, except .20C, resulted in the most abundant allele within a PCR reaction producing the larger allele peak on the pherogram. For this locus, allele size (in bp) did not seem to bias PCR amplification, except for the one case in test .20C, where the 169 allele was preferentially amplified and/or drawn up into the capillary of the Genescan instrument, resulting in a higher peak, despite that allele having a lower concentration in the PCR mixture. In summary, this suggests that for locus 306, clones that are 10x less abundant than another within the blood stream of the host can still be detected.
For locus 747, most of the experimental runs did not result in amplification of the most abundant allele within the PCR, rather, a certain larger allele, 193, was always preferentially amplified, no matter which allele it was paired with. Reduction in concentration of this allele also did not seem to have an effect on its amplification. Smaller alleles, when in weaker concentrations (2BC and .20C), were undetectable, suggesting that diversity at this locus can be extremely biased by allele size and concentration. For this locus, clonal diversity is more often than not, under estimated (this is a good reason to always use multiple loci for detecting multi-clone infections).

Locus 732, like 306, gave expected results, with the most abundant allele within a PCR reaction being preferentially amplified over alleles in lower concentration. The only discrepancy is in the 1:1 mixtures. Because PCR often amplifies smaller fragments at a higher rate than larger fragments, and because the ABI instrument preferentially draws up smaller fragments, the smaller alleles showing a higher signal when in equal concentration with a larger sized allele is expected. This, however did not occur for the tests on this locus, but did for locus 306.

1.6: Summary. For each locus, a clone or genotype of the parasite will be detected by a single peak on the pherogram (the parasite is haploid). Mixed-clone infections, which are common in nature, are readily detected on the pherograms. However, both the repeat length of an allele and the relative concentrations of the DNA of the clones will affect the ability to detect them via PCR and fragment analysis on the ABI instrument. PCR has been shown to bias certain fragment sizes, usually smaller fragments (as seen in the 306
examples). The ABI instrument also preferentially draws up smaller fragments, so it has been hypothesized that smaller alleles will be favored for replication and detection. This is not always the case, as shown above. If multiple loci are used to determine clonality, then the concentration of clones within a DNA sample can be as little as 1:10.

Another finding from these experiments is that detecting the number of clones within an infection can be hindered by the presence of clones represented by alleles very close together in size. Microsatellites often mutate via a stepwise model, in which one repeat unit is added or deleted with each new mutation. Stutter peaks, minor peaks observed before and after the main allele peak, are often ±1 repeat length, so detecting whether a stutter peak is a true allele or not can be challenging. Alleles that may be present as post-stutter peaks can be recognized, because these peaks are relatively high compared to pre-stutter peaks. Determining true alleles from pre-stutter peaks needs further calibration. Often times, pre-stutter peaks representing true alleles are very high compared to the true allele peak, so a 50% or even 75% rule can be used to rule out the possibility of calling an allele that is only stutter. This approach has not been tested and therefore, I cannot account for the accuracy. However, for the studies on *P. mexicanum*, any pre-stutter peaks are only counted as true alleles if they are at least 50% the height of the predominant peak. In conclusion, overall clonal diversity will tend to be underestimated, especially for infections with many parasite genotypes.
Test 2: Genotypes over time within an infection

Within an infection, multiple genotypes may be present, but not necessarily in the blood stream (Bruce et al. 2000). Newly invading parasites replicate for a while in the liver before entering the peripheral blood. Amplification of microsatellite loci will only work on the parasite genotypes found in the blood, so any clones in the liver and/or other organs may be missed. Also, some clones may be present in the blood, but in very low densities, too low for detection during the competitive PCR (as described above).

Examining the clonal diversity within a single infection over time will detect if (1) alleles mutate so rapidly as to change over the course of a three-month period (rendering the use of microsatellites for these studies to be limited); (2) if new alleles show up in the infection that weren’t previously detected; and (3) if alleles within an infection remain in the same relative abundance over time.

Three tests were run. For two of these, undergraduate laboratory classes in parasitology (BIOL 245, Spring 2005 and Spring 2006) participated in the laboratory duties.

2.1: Genotypes over time: Test 1

For the first test, I utilized blood dots from Sarah Osgood’s MS thesis work (Osgood and Schall 2003). I picked 4 lizards with bleed dates spanning the course of 3 months (Osgood lizard numbers 15789, 15991, 16102, 16456). For the first two lizards, the following dates were used for DNA: 6/13/01, 7/25/01, and 9/19/01. The latter two lizards had blood sample extracted from 7/10/01, 8/8/01 and 9/19/01. These DNA samples were
amplified for a rather non-polymorphic locus, PM328. I used this locus because it was highly reliable and often gave good representations of mixed vs. single genotypes. In all cases, the same alleles were present throughout the entire sampling period, but the first PCR of 16456 did not amplify well, so the data are not reliable. Only one of these infections, 15991, had >1 clone. Both clones in that infection remained throughout the period, but the density of the ‘weaker’ clone (smaller peak on the pherogram) increased as time progressed, while the clone that initially had the higher peak remained at the same density. This is difficult to determine with any accuracy, however, because the last two genotypes had peaks that were higher than the sizing chart, usually indicative of too much DNA (Figure 2.5a).

2.2: Genotypes over time: Test 2

Undergraduate students in my parasitology lab sections performed the next two tests. For the first year, 8 students followed infections of 8 different lizards, again from Sarah Osgood’s data (Osgood lizard numbers 15701, 15686, 15963, 15794, 15991, 16006, 15784, 16100). These lizards were infected with blood from 1-3 donors, suggesting that diversity should differ between them. DNA was extracted for three different dates for each lizard (depending upon the lizard bleed dates and the student's choice of dates to work with). The three dates spanned 2-3 months. Locus 328 was again utilized; 5 µl of DNA template was used and PCR products were diluted 5:45 or 5:90 depending upon whether or not a band showed up on the gel. In the group, 3 lizards had single-clone infections and the other 5 had 2-3 clones. Interestingly, the clones in the multi-clonal
infections waxed and waned in abundance and even presence over the 2-3 month period studied. In one particular case, a clone that was not strongly present early on in the infection showed up on the last date examined, becoming a dominant clone at the end of the infection period (Figure 2.5b). Two other clones observed on the first date had diminished in abundance and presence so that at the final bleed date, they were not distinguishable. A similar result was seen for a second series (Figure 2.5c).

2.3: Genotypes over time: Test 3

The second year, 9 students followed an additional 9 individual infections from the same data set (Osgood lizards 16076, 15789, 16102, 15805, 16005, 15679, 15786, 15687, 16098, 15973). Similar results as above were found. No new alleles appeared over time, but the abundance of each clone did appear to vary over the three dates examined (Figure 2.5d).

2.4: Summary: The results of these studies over time are interesting when more than two clones appear to be present, and indicate the challenge of “defining” clonal diversity for an infection. As seen in the two examples from test 2, clones rarely appeared and disappeared throughout a three-month period, but they did vary in their density over time. If these infections were watched longer than the three months studied, the clonality of the infection may differ from the previous month. A clone that decreases in its abundance in the blood stream may become undetectable if the concentration is low (see above tests of concentration vs. detection). However, in no case did a “new” clone appear that was +
one repeat change, indicating that the alleles were not rapidly mutating at these loci, rendering them stable for long-term studies.

**Test 3: Repeatability**

To test the repeatability of the genotyping results, five infected lizards were chosen and DNA was extracted from a blood sample using the DNeasy kit (Qiagen). Using 2 µl of this DNA for template, 5 separate PCRs were done using locus Pmx306. These PCRs were run on separate days with new vials of primer and water each time. After each PCR, the product was run out on a gel and then diluted to a 1:10 solution for genotyping. If a band was present on the gel for a sample, 0.5 µl of the 1/10 solution was used for genotyping, but 1 µl was used of the 1/10 product if no band appeared. Each PCR was genotyped on a separate day to account for day-to-day fluctuations in the accuracy of the ABI instrument. In all but 1 of the PCR/genotype trials, methods and results were the same as the original genotyping done months earlier (data not shown). Thus, identical results were obtained for four samplings of the infections (dates 1, 3, 4 and 5; Figures 2.6a-e). One trial (the second in the series), and a second for lizard 17963, however, produced false results. Contamination or errors in identification of the samples when run in the ABI instrument produced a pherogram with spurious alleles in all the samples for that day.
Discussion

From these tests, it is clear that up to 6 alleles can be scored from a multi-clonal infection. However, peaks falling within the stutter of other peaks, and very small peaks for low-density clones and/or for alleles that are large (large fragment length detected in the instrument), may be missed while evaluating the pherograms. One strategy to improve detection is to recognize that if an allele falls in the post stutter range (the first stutter peak after the main allele peak) it can still be detected if the first peak post-stutter has a higher peak than any pre-stutter peak. Alleles falling in the pre-stutter range, however, are not as clearly detected and a stricter cut rule, such as 50% of the height of the main allele peak, should be applied. Therefore, for some of my studies, infections were grouped using the minimal number of clones rule (1 clone, 2 clones, 3 clones, and > 3 clones). For simple counts of number of clones, the count will often underestimate the true clonal diversity. For other studies, the product of the number alleles over all loci can be used to assess the maximum number of clones within an infection, thereby allowing me to present a range of clones within infections. In addition, I only called alleles that had a fluorescence reading greater than 50 units. This decreases the chance of calling background noise as a true allele.

While following infections over time, I noticed that changes in clonal abundance was common in many infections with greater than one clone. This illustrates the importance of taking more than a single blood sample from a given infection. However, I never noticed new alleles appearing during the course of an infection, so my methods for detecting mixed-clone infections are sensitive and consistent.
Overall, the sensitivity and repeatability of these methods render the use of these developed microsatellite markers as useful tools in assessing the clonal diversity within and among infections of *Plasmodium mexicanum* (Schall and Vardo 2007).
Literature Cited


Table 2.1. List of 6 ‘donor’ lizard extractions used and their respective alleles at locus Pmx 306. Results appear in Figure 2.1.

<table>
<thead>
<tr>
<th>Lizard</th>
<th>Alleles at locus Pmx 306</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL 6</td>
<td>207</td>
</tr>
<tr>
<td>G 28</td>
<td>173</td>
</tr>
<tr>
<td>F 10</td>
<td>191</td>
</tr>
<tr>
<td>17283</td>
<td>197</td>
</tr>
<tr>
<td>18170</td>
<td>182</td>
</tr>
<tr>
<td>18152</td>
<td>194, 169</td>
</tr>
</tbody>
</table>
Table 2.2. Specific donor lizard DNA extractions and genotypes used to create 2, 3, 4 and 5 clone mixtures. Results appear in Figure 2.2.

<table>
<thead>
<tr>
<th>Clone group</th>
<th>Lizard DNA Extraction</th>
<th>Pmx 306 alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-clones</td>
<td>17283</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>17963</td>
<td>185</td>
</tr>
<tr>
<td>3-clones</td>
<td>17283</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>17963</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>17348</td>
<td>173</td>
</tr>
<tr>
<td>4-clones</td>
<td>17283</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>17963</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>17348</td>
<td>173</td>
</tr>
<tr>
<td></td>
<td>17308</td>
<td>210</td>
</tr>
<tr>
<td>5-6 clones</td>
<td>17283</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>17963</td>
<td>185</td>
</tr>
<tr>
<td></td>
<td>17348</td>
<td>173</td>
</tr>
<tr>
<td></td>
<td>17308</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td>18152</td>
<td>170, 194</td>
</tr>
</tbody>
</table>
Table 2.3. “Multiclonal infections” created from mixing DNA extraction products of 4-5 different lizards. Lizard ID, alleles and their respective height in fluorescent units (Fl. Units) at each of 3 loci are given. Results appear in Figures 2.3a-2.3c.

<table>
<thead>
<tr>
<th></th>
<th>Locus Pmx 306</th>
<th>Locus Pmx 747</th>
<th>Locus Pmx 732</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lizard</td>
<td>Allele</td>
<td>Allele</td>
<td>Allele</td>
</tr>
<tr>
<td></td>
<td>(Fl. Units)</td>
<td>(Fl. Units)</td>
<td>(Fl. Units)</td>
</tr>
<tr>
<td>17494</td>
<td>199 (7135)</td>
<td>17628 187 (1078)</td>
<td>18139 228 (1071)</td>
</tr>
<tr>
<td></td>
<td>209 (4383)</td>
<td>271 (1530)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>172 (4226)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17479</td>
<td>184 (6907)</td>
<td>17661 199 (890)</td>
<td>17281 291 (1186)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>208 (260)</td>
<td></td>
</tr>
<tr>
<td>17716</td>
<td>169 (7642)</td>
<td>17336 160 (934)</td>
<td>15254 260 (1100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17720</td>
<td>194 (&gt;4000)</td>
<td>17847 175 (1106)</td>
<td>17318 283 (1737)</td>
</tr>
<tr>
<td></td>
<td>205 (&gt;4000)</td>
<td>19534 193 (1738)</td>
<td>193 (weak)</td>
</tr>
</tbody>
</table>

33
**Table 2.4a.** Summary of the experimental setup (lizards and alleles utilized) to examine effects of allele size and concentration on detection for Locus Pmx 306. Results in Figure 2.4.

<table>
<thead>
<tr>
<th>Trials</th>
<th>Lizards</th>
<th>Alleles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 and 2</td>
<td>17963</td>
<td>184</td>
</tr>
<tr>
<td></td>
<td>17710</td>
<td>181</td>
</tr>
<tr>
<td>3 and 4</td>
<td>18170</td>
<td>171</td>
</tr>
<tr>
<td></td>
<td>17281</td>
<td>197</td>
</tr>
<tr>
<td>5 and 6</td>
<td>17283</td>
<td>197</td>
</tr>
<tr>
<td></td>
<td>17348</td>
<td>171</td>
</tr>
<tr>
<td>7 and 8</td>
<td>17308</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td>18042</td>
<td>171</td>
</tr>
</tbody>
</table>
Table 2.4b. Summary of set of trials in which five alleles were mixed (Locus Pmx306) in various concentrations. Results in bold are those that followed expectations based upon DNA concentration (expected lower amplification via PCR for lower concentrated DNA) and allele size bias (weaker signal for lower concentration is expected). Concentrations of the DNA extraction used were either full strength (non-diluted) or diluted 1/10 H2O. (Strong= highest peak, Small = lower peak, Weak= very low peak, < 100 Fl. Units)

<table>
<thead>
<tr>
<th>Trial</th>
<th>Expected Result</th>
<th>Mixture</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>184&gt;181</td>
<td>2 µl 184 + 2 µl 1/10 181</td>
<td>181 and 184 (weak signal)</td>
</tr>
<tr>
<td>2</td>
<td>181&gt;184</td>
<td>2 µl 1/10 184 + 2 µl 181</td>
<td>181 only</td>
</tr>
<tr>
<td>3</td>
<td>171&gt;197</td>
<td>2 µl 171,185 + 2 µl 1/10 197</td>
<td>171 (weak) and 197 (strong)</td>
</tr>
<tr>
<td>4</td>
<td>197&gt;171</td>
<td>2 µl 1/10 171,185 + 2 µl 197</td>
<td>Smaller 171, strong 197</td>
</tr>
<tr>
<td>5</td>
<td>197&gt;171</td>
<td>2 µl 197 + 2 µl 1/10 171</td>
<td>Weak 171, strong 197</td>
</tr>
<tr>
<td>6</td>
<td>171&gt;197</td>
<td>2 µl 1/10 197 + 2 µl 171</td>
<td>Strong 171, small 197</td>
</tr>
<tr>
<td>7</td>
<td>209&gt;171</td>
<td>2 µl 209 + 2 µl 1/10 171</td>
<td>Strong 209, weak 171</td>
</tr>
<tr>
<td>8</td>
<td>171&gt;209</td>
<td>2 µl 1/10 209 + 2 µl 171</td>
<td>Strong 171, weak 209</td>
</tr>
</tbody>
</table>
Table 2.5a. Lizards used for each locus and the alleles in the infection for each lizard.

Shown are the alleles for each locus for each infected lizard and the ‘code’ letter for each infection (see table 2.5b).

<table>
<thead>
<tr>
<th>Locus Pmx 306</th>
<th>Locus Pmx 747</th>
<th>Locus Pmx 732</th>
</tr>
</thead>
<tbody>
<tr>
<td>cod Lizard Allele (Fl. Units)</td>
<td>code Lizard Allele (Fl. Units)</td>
<td>code Lizard Allele (Fl. Units)</td>
</tr>
<tr>
<td><strong>A</strong> 19277 185 (1226)</td>
<td><strong>A</strong> 17665 187 (2027)</td>
<td><strong>A</strong> 19539 271 (1447)</td>
</tr>
<tr>
<td><strong>B</strong> 17454 193 (&gt;1200)</td>
<td><strong>B</strong> 19534 193 (1738)</td>
<td><strong>B</strong> 19534 280 (1157)</td>
</tr>
<tr>
<td><strong>C</strong> 17318 169 (&gt;1200)</td>
<td><strong>C</strong> 15335 169 (2189)</td>
<td><strong>C</strong> 19520 206 (1521)</td>
</tr>
</tbody>
</table>

Table 2.5b. Ten combinations run for each locus. Lizards and alleles “A”, “B” and “C” differ for each locus (see table 2.5a).

<table>
<thead>
<tr>
<th>Trial Label</th>
<th>Contents</th>
<th>Trial Label</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>2AB</td>
<td>2.5 µl A + 2.5 µl 1/10 B</td>
<td>1B1C</td>
<td>2.5 µl B + 2.5 µl C</td>
</tr>
<tr>
<td>2BA</td>
<td>2.5 µl B + 2.5 µl 1/10 A</td>
<td>.20B</td>
<td>2.5 µl A + 2.5 µl 1/5 B</td>
</tr>
<tr>
<td>2BC</td>
<td>2.5 µl B + 2.5 µl 1/10 C</td>
<td>.20A</td>
<td>2.5 µl B + 2.5 µl 1/5 A</td>
</tr>
<tr>
<td>2CB</td>
<td>2.5 µl C + 2.5 µl 1/10 B</td>
<td>.20C</td>
<td>2.5 µl B + 2.5 µl 1/5 C</td>
</tr>
<tr>
<td>1A1B</td>
<td>2.5 µl A + 2.5 µl B</td>
<td>.20BC</td>
<td>2.5 µl C + 2.5 µl 1/5 B</td>
</tr>
</tbody>
</table>
Table 2.5c. Results for trials summarized in Tables 2.5a and 2.5b. For each cell, the size of the peak seen on the pherogram (large, medium, small, or no peak seen) and the allele(s) seen are given. Bolded cells represent assays that followed expectations.

<table>
<thead>
<tr>
<th>Label</th>
<th>Locus 306</th>
<th>Locus 747</th>
<th>Locus 732</th>
</tr>
</thead>
<tbody>
<tr>
<td>2AB</td>
<td>Large 184, Small 193, 199</td>
<td>Med 187, Large 193</td>
<td>Large 270, small 280</td>
</tr>
<tr>
<td>2BA</td>
<td>Small 172, 184, Large 193, 199</td>
<td>Small 187, Large 193</td>
<td>No 270, Large 280</td>
</tr>
<tr>
<td>2BC</td>
<td>Med 169, Large 193, small 199</td>
<td>No 169, Large 193</td>
<td>Small 206, Large 280</td>
</tr>
<tr>
<td>2CB</td>
<td>Large 169, Small 193</td>
<td>Small 169, Large 193</td>
<td>Large 206, Small 280</td>
</tr>
<tr>
<td>1A1B</td>
<td>Large 184, Small 193, 199</td>
<td>Small 187, Large 193</td>
<td>Small 270, Large 280</td>
</tr>
<tr>
<td>1B1C</td>
<td>Large 169, Small 193, 199</td>
<td>Small 169, Large 193</td>
<td>Small 206, Large 280</td>
</tr>
<tr>
<td>.20B</td>
<td>Small 172, Large 184, med 199</td>
<td>Med. 187, Large 193</td>
<td>No show</td>
</tr>
<tr>
<td>.20A</td>
<td>Small 172, 184, Large 193, 199</td>
<td>Small 187, Large 193</td>
<td>Small 270, Large 280</td>
</tr>
<tr>
<td>.20C</td>
<td>Large 169, Small 193, 199</td>
<td>No 169, Large 193</td>
<td>Small 206, Large 280</td>
</tr>
<tr>
<td>.20BC</td>
<td>Large 169, Small 193</td>
<td>Small 169, Large 193</td>
<td>Large 206, Small 280</td>
</tr>
</tbody>
</table>
**Figure Captions**

**Figure 2.1.** A pherogram resulting from mixing 7 known clones (alleles) of *P. mexicanum*. Without prior knowledge of the clonal diversity present, only four peaks would have been identified (indicated with arrows). The other three alleles fell into pre- and post stutter ranges, indicated by dashed arrows.

**Figure 2.2.** Calibration test 1b: Detecting multiple peaks with varying allele sizes. For the 2, 3, and 4-clone mix, all alleles were clear, although the height of the peaks varied substantially. For the 5-6 clone mix, only 4 alleles were clear because two clones fell within the stutter of another allele. (Allele 185 was very small, and could only be seen when the pherograms were expanded by the GeneMapper software. dashed arrows represent alleles falling in pre-stutter range).

**Figure 2.3.** Calibration test 2: detection of multiple alleles within a single infection. (a). Combination of alleles for Locus Pmx306 that differed in size such that the true peaks and stutter would not overlap. Six alleles (clones) were included in the mixture of template DNA, and as seen on the pherogram, four peaks are clear. Alleles (clones) falling within the stutter range are represented by dashed arrows. (b). A pherogram for Locus Pmx747 for a mixture of template DNA known to contain 6 alleles (clones). Note that four clones are obvious. The dashed arrow represents the alleles not amplified. (c). Pherogram for Locus Pmx732, with a known mixture of 5 alleles. Only two weak alleles are obvious.
**Figure Captions**

**Figure 2.4.** Calibration test 3: Detection of clones with varying sizes and concentrations. Dashed arrows represent alleles that did not amplify. Bolded results followed expectations.

**Figure 2.5a-2.5d.** Calibration test: Genotypes over time. Pherograms for four infections followed each month over a three-month period. Notice the change in allele peak heights and presence over time.

**Figures 2.6a-2.6e:** Calibration test: Repeatability assay. Each of five lizards was genotyped at locus Pmx 306 on five different days (1-5), and analyzed at the Cancer Facility by date. In all cases except for lizard 17336, date “2” had contamination issues—different alleles are shown that are not detected in other trials. Additionally, lizard 17963 had a similar genotype result on date 4 and on date 2.
Figure 2.1

![Figure 2.1](image1)

Figure 2.2

![Figure 2.2](image2)
Figure 2.3
Figure 2.4

Trial 1

184>181

Trial 2

181>184

Trial 3

197>171

Trial 4

197>171

Trial 5

197>171

Trial 6

171>197

Trial 7

209>171

Trial 8

171>209
Figure 2.5c

Figure 2.5d
Figure 2.6a:
Lizard 17281
Figure 2.6b

Lizard 17336
Figure 2.6c

Lizard 17963
Figure 2.6d

Lizard 18064
Figure 2.6e

Lizard 18152
Chapter 3

PCR detection of lizard malaria parasites: prevalence of Plasmodium infections with low-level parasitemia differs by site and season

Anne M. Vardo, Andrew R. Wargo, and Jos. J. Schall

Abstract

*Plasmodium*-specific polymerase chain reaction (PCR) primers allowed detection of infections with very low-level parasitemia for 3 species of malaria parasite infecting *Anolis* lizards at 2 Caribbean sites, Puerto Rico and Saba, Netherlands Antilles. A verification study, using a single tube nested PCR to eliminate contamination, showed that infections as low as 1 parasite per millions of erythrocytes could be detected by amplifying a 673 bp fragment of the cytochrome b gene. Very low-level parasitemia infections, subpatent under the microscope, were common in *A. sabanus* on Saba sites, with no seasonal difference (31% of infections appearing uninfected by microscopic examination in summer were found infected by PCR, 38% in winter). At the Puerto Rico site, the subpatent infections were also common in *A. gundlachi*, but were more prevalent in winter (53%) than in summer (17%). A similar high frequency of subpatent infections is known from studies on human and bird malaria, but a previous PCR-based study on a temperate lizard malaria system found few such low-level infections. Differences in the prevalence of subpatent infections by site and season suggest transmission biology may select for distinct life history strategies by the parasite.
Introduction

The prevalence of malaria parasites (*Plasmodium*) in their vertebrate host populations has traditionally been determined by microscopic examination of blood smears. Parasitologists have long known that this is an inaccurate method because many infections are in the subpatent phase with low-level parasitemia, resulting in these infections being falsely scored as negatives. Examination of multiple smears taken over time from suspected infected hosts is the classic method to correct for this error (Macdonald, 1926; Herman, 1939). Two modern techniques, polymerase chain reaction (PCR) amplification of parasite DNA and serological tests, allow a more accurate estimate of infection prevalence. The PCR detects extremely low-level infections in the blood, far below the parasitemia needed for success, even if multiple slides are scanned (Perkins *et al.*, 1998; Richard *et al.*, 2002; Hellgren *et al.*, 2004). However, PCR will fail to detect infections located only in deep tissues or later infections that have ceased parasite replication in the blood. Serological techniques are more precise in such cases (Jarvi *et al.*, 2002), but may produce false positives for infections that have been cleared from the host.

Although PCR provides an imperfect estimate of the actual prevalence of malaria parasites, the technique may allow insight into the life history ecology of *Plasmodium* spp. For example, previous PCR studies demonstrated that the proportion of false negatives (negative after scanning smears, but positive by PCR) can be high, up to 67% for *Plasmodium* spp. infections of humans, but lower in birds (about 25% of birds sampled) (reviewed in Perkins *et al.*, 1998; Richard *et al.*, 2002), and very low in a study
of the lizard malaria parasite, *P. mexicanum*, at a California site (only 4-6% of the samples were false negatives) (Perkins *et al.*, 1998). The lizard malaria study in California also found differences in the prevalence of subpatent infections by site, with a high proportion of all infections being low-level at a site with low overall prevalence, and rare at a site where overall prevalence was high. Thus, very low-level parasitemia infections were far more common where transmission may be less intense. This suggests that such infections may be a life history strategy by the parasite when faced with times or places with rare or even impossible transmission, and in such cases the parasite may reduce its rate of replication or parasitemia to insure minimal cost to its host (Gill and Mock, 1985; Ewald, 1994).

Here, we report the proportion of subpatent infections detected by PCR for lizard malaria parasites at 2 tropical sites, a rain forest in eastern Puerto Rico and on the tiny island of Saba, Netherlands Antilles. *Anolis gundlachi* in Puerto Rico and *A. sabanus* on Saba are infected with the same 3 species of *Plasmodium*: *P. floridense* and 2 species now combined under *P. azurophilum* (Staats and Schall, 1996; Schall *et al.*, 2000). The *P. azurophilum* complex includes 2 morphologically identical parasites, one infecting erythrocytes, and the other infecting several classes of white blood cells (Perkins, 2000; 2001).

Prevalence is slightly higher in Puerto Rico compared to the California lizard malaria system (20-45% among sites and males vs. females in Puerto Rico), and substantially higher on Saba (50-70% at sites used in this study) (Staats and Schall, 1996; Schall *et al.*, 2000). Examination of blood smears of samples taken over the period 1992-2002 on
Saba revealed no seasonal variation in prevalence of the parasites. However, apparent prevalence between 1996 and 2002 at the Puerto Rico site was consistently lower in the winter vs. summer (Staats and Schall, 1996; Schall et al, 2000; data not shown). This change in apparent prevalence was observed even for samples taken 5 mos apart, when the actual proportion of lizards infected was unlikely to have changed.

These results suggest that the ecology of the parasite-host association, including transmission biology, differs among the 2 tropical sites and the temperate California system. If differing transmission ecology selects for distinct life history strategies by the parasites, the occurrence of very low-level infections should differ among sites and seasons in the Caribbean even though the same 3 parasite species are present. We use the results of a PCR study to test this prediction.

Methods

The study sites were the El Verde Field Station within the Luquillo Mountains in northeastern Puerto Rico (Waide and Reagan, 1996; Schall et al., 2000) and several sites near the town of The Bottom on Saba, Netherlands Antilles (Staats and Schall, 1996). Lizards were collected by hand or slip noose on a fishing pole, and a blood sample taken from a toe clip. Blood drops were used to make thin smears (to be stained with Giemsa) and dried dots on filter paper. Samples used in this study were taken from 1996 to 1999 on Puerto Rico and 1997 to 1999 on Saba.

DNA was extracted from dried blood dots using the DNeasy kit (Qiagen, Valencia, CA) and the provided protocol, except only 30 ml of elution buffer was used during the
final stage. PCR primers specific for the *Plasmodium* cytochrome b gene were used in a nested set of reactions. An outer reaction used primers DW2 (5' TAA TGC CTA GAC GTA TTC CTG ATT ATC CAG 3') and DW4 (5' TGT TTG CTT GGG AGC TGT AAT CAT AAT GTG 3') with 1 ml of each 10 mM primer and 2 ml of extracted DNA in a 25 ml PCR reaction using Ready-to-Go beads containing dNTP, *taq* polymerase, and buffers (Amersham, Piscataway, NJ). Reaction conditions were a preliminary step of 94 C for 4 min, followed by 35 cycles of 94 C for 20 sec, 60 C for 20 sec, 72 C for 90 sec, with a final step of 7 min at 72 C. A second reaction used 1 ml of the first PCR product and primers DW1 (5' TCA ACA ATG ACT TTA TTT GG 3') and DW3 (5' TGC TGT ATC ATA CCC TAA AG 3'), and reaction conditions of a preliminary step of 94 C for 1 min, followed by 40 cycles of 94 C for 20 sec, 50 C for 20 sec, 72 C for 90 sec, with a final step of 7 min at 72 C. Extreme care was taken to prevent contamination during the second reaction, and a control known positive and negative (water replacing DNA) was included in each set of reactions. Presence of the 673 bp product from the second reaction was visualized on a 1% agarose gel. The primers amplify DNA from all three species of *Plasmodium* found at the two sites. This was determined by amplifying solitary infections and sequencing the DNA fragment (Perkins 2000, 2001).

Two kinds of verification runs were performed to determine the sensitivity of the PCR technique. First, known infected lizards (known positives) were chosen and their parasitemia determined by counting parasites seen in 1,000 erythrocytes. These samples were then assayed using the above nested PCR reactions. A second verification method eliminated an important source of contamination during nested PCR. Preliminary results
revealed an apparent high proportion of false negatives in the sampled lizards, so we wished to eliminate the possibility that contamination during the transfer of PCR product from the first reaction to the next PCR was presenting spurious results. Therefore, we extracted DNA from a known infected lizard, then diluted the DNA to determine the lowest concentration when the nested method detected the parasite. Contamination was prevented during this procedure by using a modification of the single tube nested PCR method of Abath et al. (2002). Briefly, each inner primer (DW1 and DW3) was diluted to 1 mM, and 10 ml of each primer was put inside the cap of a 0.5 ml vial and allowed to dry. The two outer primers (DW2 and DW4) were diluted to 0.01 mM concentration. A PCR mixture was prepared as described above for an outer reaction, then 25 ml gently pipetted into the vial with the dried inner primers on its lid, and 20 ml of oil was placed on the surface of the reaction mixture. The vial was closed and placed into a PCR thermalcycler with its heated lid turned off. The outer PCR reaction was used, but with only 10 cycles. The reaction mixture was allowed to cool to 15 C, and then the vial was shaken vigorously to mix the inner primers into the mixture. The second PCR (inner reaction conditions) was then run. The low concentration of outer primers allowed them to be completely consumed in the first reaction. Again, the PCR product was visualized on a 1% agarose gel. The success of the single tube PCR method in detecting extremely low concentrations of parasite DNA argued that the high proportion of false negatives discovered was not a result of contamination (below). We, therefore, reverted to the 2 tube method for the study, but used extreme caution when opening tubes and set up reactions under a UV hood to destroy errant DNA.
Blood samples from lizards scored as negative after a 6 min scan of blood smears under 1,000 x magnification were used for template DNA in the nested PCR protocol. Any samples showing a positive PCR reaction (thus a false negative under the microscope) led to a second examination of the blood smear to determine if any errors were made when scanning slides. A few such cases were found, and these samples were placed into the known positive data category. That is, the study's purpose was not to determine the accuracy of the slide scanning, but to find low-level infections that were not obvious under the microscope.

**Results and Discussion**

All 83 known positives (38 from Saba and 45 from Puerto Rico) produced product readily observable on the gel. Of these, 50 were from infections with parasitemia from < 1 to 5 parasites per 10,000 erythrocytes (32 *P. azurophilum* [erythrocyte-infecting species], 6 *P. azurophilum* [species in white blood cells], 7 *P. floridense*, and 5 that could not be identified to species). An infection with parasitemia of 314/10,000 erythrocytes was diluted from 1x to 0.000001x for use in the single tube nested PCR. All dilutions produced a band on the agarose gel, although a bright band was seen only to a dilution of 0.001x (Fig. 3.1). Thus, an infection with parasitemia of 1 parasite per 30 x 10^6 erythrocytes can be detected by the assay.

False negatives (negative by slide scanning but positive by the PCR assay) were common for both Puerto Rico and Saba. On Saba, 38% (N = 40) of winter samples and 31% (N = 48) of summer samples proved to be false negatives, with no difference
between winter and summer ($\chi^2=0.379, P=0.538$). On Puerto Rico, false negatives were more common in winter than summer, 53% (N = 30) in winter and 17% (N = 46) in summer ($\chi^2=10.85, P=0.001$). There was no difference in proportion of false negatives for the summer samples comparing the islands ($\chi^2=1.174, P=0.187$), but differed for the winter samples ($\chi^2=3.93, P=0.047$).

The PCR method proved a highly sensitive assay for very low-level parasitemia infections. The dilution trial demonstrated that an infection can be detected with parasitemia of 1 parasite per millions of red blood cells. Our 6 min scanning protocol allows examination of approximately 10,000 cells. To detect such a very low-level infection would require scanning multiple smears for 300 hr. Richard et al. (2002) similarly found that a nested PCR for cytochrome b was very sensitive for detection of avian malaria infections.

Very low-level parasitemia infections were common on both Caribbean islands, similar to the reported results for human and bird malaria, but subpatent infections were far more common than found in the study of a temperate lizard malaria system. False negatives after microscope scanning were only 4-6% of samples in California, but 17-53% for the 2 tropical locations. This overall lower parasitemia for the Caribbean lizard malaria parasites may account for the lack of virulence observed for these species compared to the high virulence seen for P. mexicanum infections (Schall and Pearson, 2000; Schall, 2002; Schall and Staats, 2002).

Infections at the earliest stages, when parasites have just entered the blood, would be subpatent under the microscope. Some of the infections found by PCR on the tropical
islands likely fell into this category, but we frequently recapture infected lizards on both Puerto Rico and Saba after several mo or a yr, so it is highly unlikely that the 2 Caribbean sites experience a turn-over of infections high enough to account for the high proportion of very weak parasitemia infections observed there. A more likely explanation is that the parasite follows a life history strategy that keeps replication and parasitemia low for some sites and seasons. Transmission at the California site is strongly seasonal, and parasitemia drops during the winter when lizards are dormant, to rebound again the next spring (Bromwich and Schall, 1986; Eisen, 2000). High parasitemia may be optimal for infections during the short transmission period, especially if many infections contain competing clones of parasites. In contrast, lizards are active yr-round at the tropical sites and transmission may be far less seasonal on the islands. However, the sharp increase in low-level infections during winter in Puerto Rico suggests that transmission there may be reduced during that period.

One other important difference between the temperate and tropical study systems is the presence of 3 Plasmodium species in the Caribbean, and only 1 in California. There is no way at this time to determine if subpatent infections are more common for 1 or 2 of the 3 species on Puerto Rico or Saba. That is, the positive result on the PCR analysis does not provide information on which species was or were being detected. Theoretical discussions of the life history strategies of parasites typically revolve around transmission biology, such as seasonality or vector abundance, with less discussion on the genetic structure of the infections (but see Eisen and Schall, 2000). The role of interspecific interactions of malaria parasites on infections is even more rarely examined (Schall and
Bromwich, 1994). The role of interspecific interactions among species of Caribbean lizard malaria remains an open question. For example, the 3 *Plasmodium* species infecting *Anolis* spp. on Caribbean islands may well drop to low levels as they compete within an individual host, perhaps through manipulation of the host immune system.

**Acknowledgements**

We thank the staff of the El Verde Field Station for logistical assistance and laboratory space, and the people of Saba, especially T. van't Hof and H. Cornet of Saba Ecolodge, for their assistance and warm welcome. T. C. Smith and S. M. Osgood helped in the field and T. C. Smith assisted with the laboratory duties. The research was funded by a grant from the National Science Foundation, and conducted under approval given by the El Verde Field Station and the government of the Netherlands Antilles and an approved animal use protocol by the University of Vermont.
Literature Cited


Figure Captions

Figure 3.1. 1% agarose gel showing PCR product obtained by single tube nested PCR for a 673 bp segment of the cytochrome b gene of *Plasmodium*. DNA was extracted from an infected lizard, then diluted from 1x to 0.000001x. Bands are seen for all dilutions, but weak bands for 0.001 or lower concentration of the extracted DNA.
Figure 3.1
Chapter 4
Clonal diversity of a lizard malaria parasite, *Plasmodium mexicanum*, in its vertebrate host, the western fence lizard: role of variation in transmission intensity over time and space

A. M. Vardo and J. J. Schall

**Abstract**
Within the vertebrate host, infections of a malaria parasite (*Plasmodium*) could include a single genotype of cells (single-clone infections) or two to several genotypes (multiclone infections). Clonal diversity of infection plays an important role in the biology of the parasite, including its life history, virulence, and transmission. We determined the clonal diversity of *P. mexicanum*, a lizard malaria parasite at a study region in northern California, using variable microsatellite markers, the first such study for any malaria parasite of lizards or birds (the most common hosts for *Plasmodium* species). Multiclonal infections are common (50 - 88% of infections among samples), and measures of genetic diversity for the metapopulation (expected heterozygosity, number of alleles per locus, allele length variation, and effective population size) all indicated a substantial overall genetic diversity. Comparing years with high prevalence (1996 to 1998 = 25 - 32% lizards infected), and years with low prevalence (2001 to 2005 = 6 - 12%) found fewer alleles in samples taken from the low prevalence years, but no reduction in overall diversity (H = 0.64 - 0.90 among loci). In most cases, rare alleles appeared to be lost as prevalence declined. For sites chronically experiencing low
transmission intensity (prevalence ~ 1%), overall diversity was also high (H = 0.79 – 0.91), but there were fewer multiclonal infections. Theory predicts an apparent excess in expected heterozygosity follows a genetic bottleneck. Evidence for such a distortion in genetic diversity was observed after the drop in parasite prevalence under the infinite alleles mutation model but not for the stepwise mutation model. The results are similar to those reported for the human malaria parasite, \textit{P. falciparum}, worldwide, and support the conclusion that malaria parasites maintain high genetic diversity in host populations despite the potential for loss in alleles during the transmission cycle or during periods or in locations where transmission intensity is low.

\textbf{Introduction}

Within their vertebrate hosts, malaria parasites (\textit{Plasmodium}) replicate asexually as clones of haploid cells, and each infection may consist a single genotype of cells (single-clone infection), or several to many genotypes (multiclone infection) (Babiker \textit{et al.} 1999). Theory argues that the clonal diversity of infections should play a central role in the biology of microparasites such as \textit{Plasmodium} (Griffin & West 2002; Foster 2005), but data have long been lacking to test this view (Read & Taylor 2001). In the case of \textit{Plasmodium}, though, evidence is growing that genetic diversity is indeed important for competitive interactions among clones (deRoode \textit{et al.} 2005), virulence (Smith \textit{et al.} 1999; Read & Taylor 2001), facilitation (positive interaction) (Gilbert \textit{et al.} 1998), premunition (prevention against superinfection) (Smith \textit{et al.} 1999; Vardo \textit{et al.} 2007), and degree of inbreeding and optimal sex allocation (Read \textit{et al.} 1992). Data on clonal
diversity among infections also provides insight into population structure (Konaté et al. 1999; Awadalla et al. 2001) and the transmission dynamics within and among sites (Paul et al. 1998, Anderson et al. 2000; Ferdig & Su 2000). Thus, assessing the clonal diversity of Plasmodium, both within and among infections, is necessary for any understanding of the ecology of these diverse parasites.

The number of clones within malaria infections must depend in part on the prevailing genetic diversity of the parasites among all infections (the parasite's metapopulation). Tibayrenc et al. (1991) and Tibayrenc and Ayala (2002) noted that, despite the obligate sexual cycle in Plasmodium parasites, P. falciparum in human populations at some locations may have little clonal diversity or even a uniclonal population structure. Malaria parasites could lose genetic diversity if not all clones are successful during the transmission cycle, or if competition within infections excludes clones. During times or at locations experiencing low transmission intensity, overall clonal diversity could decline. Empirical studies on clonal structure of Plasmodium infections of humans reveal multiclonal infections are common at locations where transmission is intense (many vectors biting), with lower clonal diversity where transmission is rare (Paul et al. 1995; 1998; Anderson et al. 1999; 2000; Babiker et al. 1999; 2000; Ferreira et al. 2002; Abdel-Muhsin et al. 2003; Cui et al. 2003; Durand et al. 2003; Imwong et al. 2006; Zakeri et al. 2006). Although these results indicate a substantial range of genetic structure among geographic locations, the results are confounded by rapid movement of humans among geographic sites, widespread bed net use, and selective sweeps experienced by human malaria parasites caused by antiparasite drug use (Nair et al. 2003). Data on clonal
diversity for malaria parasites of nonhuman vertebrate hosts (reptiles, birds, and nonhuman mammals) are not available. These systems, though, may provide a more clear picture of how the life cycle itself may determine the genetic structure of the parasites.

Here we use recently characterized variable microsatellite loci for the lizard malaria parasite, *P. mexicanum* (Schall & Vardo 2006), to determine the clonal diversity both within and among infections over years and among sites. This system is particularly useful for such studies because prevalence of *P. mexicanum* in its natural lizard host, the western fence lizard *Sceloporus occidentalis*, has waxed and waned over a 28 year period at our long-term study location in northern California, USA. Also, there are sites at the study location where infection prevalence has been chronically high or low; that is, even as overall prevalence has changed over the years, some sites always had the highest prevalence and some maintained consistently much lower prevalence. Our goals were (1) To determine the distribution of number of clones among infections of *P. mexicanum* using microsatellite markers. These data will reveal if vector-borne parasites such as *Plasmodium* can maintain a high clonal diversity in a host with little host mixing among geographic locations. (2) To compare clonal diversity of *P. mexicanum* for a period of years when prevalence of the parasite was high vs. another period when prevalence was low. Such sudden drops in prevalence appear common for malaria parasites of both human and nonhuman vertebrate hosts (Schall & Marghoob 1995; Babiker et al. 2000), so the data can reveal if these changes in prevalence are associated with a reduction in genetic diversity for the parasite metapopulation. (3) To examine clonal diversity at sites
with consistently low prevalence to determine if long-term weak transmission intensity will result in very low clonal diversity for the parasite. We then compare our results with those reported for human malaria parasites over a worldwide distribution. Thus, we can compare the clonal diversity seen in human malaria parasites, systems that have been greatly disturbed by population mixing and interventions to reduce transmission and infection, with an undisturbed system in a nonhuman host.

**Methods**

*Study areas*

The study area was a 2,169 ha tract of grass-oak woodland at the Hopland Research and Extension Center ("Hopland") 6.4 km NE from the town of Hopland in southern Mendocino County, California (Schall 1996). The climate at Hopland is hot and dry in the summer, and cool and rainy in winter, with transmission of *P. mexicanum* ceasing during the cool months. *P. mexicanum* infects only the fence lizards at the site, and two species of sandflies (psychodidae) are the insect vectors (*Lutzomyia vexator* and *L. stewardii*) (Fialho & Schall 1995). Each warm season between 1978 to 2005 fence lizards have been collected from sites scattered over the property, and blood smears made for microscopic examination. Since 1996, dried blood samples have been stored frozen for genetic studies.

Prevalence of the parasite (percent of lizards infected) differs among local sites, with high- and low-prevalence sites often nearby. This pattern has remained for many years (Schall & Marghoob 1995; Eisen & Wright 2001 and subsequent unpublished data). We
chose nine sites that maintained the highest relative prevalence over the 28-year study period, and five sites that consistently had the lowest prevalence over time (of course, excluding sites where malaria was always absent). The high prevalence sites (each 1 - 3 ha) were within 1.5 km of one another. The low prevalence sites (3 - 10 ha) were more scattered at higher elevations and were within 3.2 km distance of one another. High and low prevalence sites are 1.2 to 4.4 km distant from one another. All sites were within 4.4 km of 39° 00’ 22” N, 123° 05’ 13” W. Mark-recapture studies demonstrate lizards remain in a very local area for years (perhaps their entire adult lives) (Bromwich & Schall 1986; Eisen 2000). Prevalence also varied over the 28-year period. Approximately 20,000 lizards were sampled over the nearly three decades to document such changes in prevalence. During high prevalence years (1978-1999) at the high prevalence sites, 25 - 32% of lizards were infected (combining all sites); this prevalence dropped in later years (2001-2005) to 6 - 12% (combining all sites). (No sample was taken in 2000.) During years with higher prevalence at the low prevalence sites, < 1% to 2% of lizards were infected, with prevalence dropping in the low prevalence years (<1%).

Here, clonal diversity is compared for several samples: (1) HiYrs. This sample included infections from the nine high prevalence sites for a series of years with high prevalence (1996 – 1998). These years followed a long period (from 1978) when prevalence was highest at the field site (above). (2) LowYrs. Again, these were infections from the nine high prevalence sites, but only for a set of years with relatively low prevalence (2001 – 2004). (3) LowestYr. Infections are included from the nine high prevalence sites, but for the most recent low prevalence year (2005). If clonal diversity is
Molecular methods

For assessing overall clonal diversity, and to compare diversity measures for the high prevalence and low prevalence times and sites, the number of clones per infection was assayed using three microsatellite markers, Pmx306, 732, and 747 (Schall & Vardo 2007). To detect any distortion in genetic diversity after the possible bottleneck, an additional three loci were scored for the LowYrs and LowestYr samples, Pmx328, 710, and 839. All the loci contain a three-base repeat (ATT). DNA from dried lizard blood was extracted using the DNeasy kit (Qiagen), and the microsatellite loci amplified by PCR using one primer labeled with a fluorescent marker. All primers and PCR conditions are given in Schall and Vardo (2007). PCR product was run on an ABI 3130x1 Genetic Analyzer and the resulting pherograms examined using GeneMapper v3.7 (2004, ABI).
Each peak on the resulting pherogram represents a single clone of haploid parasites (length allele).

Infections were chosen from lizards collected from the high prevalence sites during high prevalence years of 1996, 1997, and 1998 (the final years of this high prevalence period for which we have dried blood samples), and from the following low prevalence years of 2001, 2002, 2003, 2004 and 2005. Thus, the first set of infections were collected after a long period of relatively higher prevalence, whereas the samples from the lower prevalence years were from 1 to 6 years after the proportion of lizards infected dropped. Infections from chronically low prevalence sites were sampled only during 1996-1998.

**Measures of clonal diversity**

Several measures of clonal diversity were calculated. In diverse infections, or infections with low density of one or more clones in the blood, some peaks on the pherograms may be too low to be scored. In preliminary trials we mixed DNA from single-clone infections that produced similar peak heights on the pherograms, and found that if one clone with a larger allele size (longer repeat) was < 10% the density of the other, it would often be missed on the pherogram. Thus, low-level infections of a clone could be missed, and overall genetic diversity could be biased low. However, up to six clones could be detected when the DNA was mixed in approximately equal proportions prior to PCR. The problem of missing clones in low density is present for any study of clonal diversity in malaria infections, so we followed the methods originally used by Anderson et al. (2000), and followed in subsequent studies (Ferreira et al. 2002; Bruce et
al. 2006). Using these methods also allow comparisons of results for *P. mexicanum* with previous studies on human malaria. To determine allelic diversity for each locus, two criteria were used when reading pherograms resulting from the GeneMapper software: highest peak (strongest signal), and all peaks 1/3 the height of the highest peak. For comparisons of clonal diversity within individual infections, all peaks were scored that were above 50 fluorescent units on the pherogram. Peaks were scored only if they demonstrated a standard shape (stutter pattern). Because the parasite is haploid in the lizard blood, each peak represents a clone for that locus. The total possible clones for each infection would be the product of the total for each locus, but this value must greatly overestimate the true clonal diversity. Therefore, we report clonal diversity within an infection as number of clones for the largest number of clones seen for any locus.

A population-wide measure of overall genetic diversity in the parasite metapopulation among infections is given using several metrics. The total number of alleles and the maximum difference in length are reported for each locus. We also report a genetic diversity metric as \( H = \frac{n}{n-1} (1 - \sum p_i^2) \) where \( n \) is the number of infections and \( p_i \) is the frequency of the \( i^{th} \) allele (the expected heterozygosity of Anderson et al. (2000a). The 95% confidence intervals for \( H \) (based on 10,000 replicate \( H \) values) were constructed using a bootstrap method described by Dixon (1993). Using the estimate of mutation rates for microsatellites obtained for *P. falciparum* \( (1.59 \times 10^{-4}) \) (Su et al. 1999), we calculated the effective population size assuming a stepwise mutation model: \( N_{e,m} = \frac{1}{8} \left\{ \frac{1}{(1-H)^2} - 1 \right\} \) (Anderson et al. 2000). During a population bottleneck, rare alleles will be lost before genetic diversity is reduced, resulting in a transient excess in
diversity over what is expected under a mutation-drift equilibrium (Nei et al. 1975). To determine if such a distortion in genetic diversity measures occurred after the drop in prevalence, we used the method of Cornuet and Luikart (1996) and the BOTTLENECK program (Piry et al. 1999).

Results

Clonal diversity within infections

Multiclonal infections were common for all three loci (Table 4.1). Combining all loci and samples, 50 - 88% of infections contained more than one clone among the samples. Comparisons of clone numbers per infection and numbers of multiclonal infections reveals a significant difference among samples ($\chi^2 = 37.8$, $P = 0.0002$, $df = 12$ for number of clones and $\chi^2 = 25.8$, $P < 0.0001$, $df = 3$ for number of multiclonal infections), but a posthoc cell contributions test demonstrated this effect was driven only by the relatively low diversity of infections at the low prevalence sites ($P < 0.05$ for only that cell). That is, whereas 78 – 88% of infections were multiclonal for the high prevalence sites for all samples, only 50% of infections were multiclonal for the chronically low prevalence sites (Table 4.1; Fig. 4.1).

Overall clonal diversity

Table 4.2 presents measures of clonal diversity among infections. Despite repeated efforts, some samples proved refractory to amplification for some loci. Comparing number of alleles per locus, size range (repeat number), and expected heterozygosity for
the two methods of scoring alleles (highest peak only vs. all peaks 1/3 height of highest peak on the pherogram) shows either a slightly lower estimate of diversity when scoring only the highest peak, or a very similar result.

After the prevalence of *P. mexicanum* dropped from the HiYrs to the LowYrs and LowestYr samples, the number of alleles scored in the samples declined. For example, at one locus (Pmx 732), more than half of the alleles present during the high prevalence years were absent by 2005. Also, fewer alleles were found at the low prevalence sites. For all loci and all time/site comparisons, 30 alleles were absent after the reduction in prevalence, half were alleles at an original frequency of 1% or less, and 90% were at a frequency of 5% or less in the parasite metapopulation among all infections (Table 4.3). Only one common allele was absent after the decline in prevalence, one of the Pmx732 alleles that was 11% frequency during the HiYrs, was absent in the sample by the LowYrs. The loss of scored alleles from high prevalence to low prevalence years may reflect a real reduction in frequency or even loss of those alleles in the parasite metapopulation during a genetic bottleneck. Alternatively, rare alleles may simply not be sampled even if they did not change in their frequency. The probability of an allele of frequency of p being missed, by chance, in a sample of n is \((1-p)^n\). Of the 30 alleles possibly lost in the parasite metapopulation, 20 had a > 5% probability of being missed in the later sampling (range 6% to 26%) if their frequency remained the same as during the high prevalence years. However, picking the 11 alleles with the highest probability of being missed (> 25%), the probability of missing all is \(3.2 \times 10^{-7}\) (product of the 11 probability values). It is therefore highly likely that some of the alleles, perhaps most,
were actually lost in the parasite metapopulation. Also, no new alleles were detected in the low prevalence samples.

The reduction in number of alleles scored after prevalence declined was not associated with a reduction in overall diversity (H). Fig. 4.1 shows substantial overlap of confidence intervals for H estimated for the three loci for all samples.

Theory and simulations reveal that a substantial population bottleneck will result in a short-term increase in genetic diversity compared to that expected under a mutation-drift equilibrium based on the number of alleles present (Nei et al. 1975; Cornuet & Luikart 1996). A minimum of six loci is required to test for this distortion after a possible bottleneck. Comparing observed genetic diversity (expected heterozygosity based on actual clonal frequencies) and heterozygosity at equilibrium for the number of alleles (BOTTLENECK program), we found a significant excess in expected heterozygosity under the infinite alleles model of mutation (IAM) in which any length allele can mutate to any other length, but no evidence of an excess under the stepwise mutation model (SMM) in which any length allele can mutate only to an allele one repeat longer or shorter (Table 4.4).

**Discussion**

In contrast to the situation for human malaria parasites, the genetic structure of *P. mexicanum* has not been disturbed by substantial population mixing over broad geographic areas, human intervention to reduce transmission, or selective sweeps initiated by drug treatment. Thus, the clonal diversity patterns of *P. mexicanum* should be
a product strictly of events during the parasite’s life cycle and changes in transmission intensity and prevalence driven by environmental or endogenous factors. This parasite system therefore provides a clear test of the view that malaria parasites should have reduced diversity because of loss of clones during transmission, loss of diversity in areas with low transmission intensity, or bottlenecks during periodic low transmission periods.

We scored clonal diversity for *P. mexicanum* at three microsatellite loci over a 3-year period ending a much longer period with high prevalence (from 25 to 32% of lizards infected from 1978 to 1998). Prevalence subsequently dropped from 2001 to 2005, and we scored clonal diversity for 2001 – 2004, and then 2005, the year with lowest prevalence (6%). These samples were all taken at sites where prevalence of the parasite has been highest at the study region. A set of other sites, that maintained very low prevalence (1% or lower) over the years was also sampled. The results reveal that *P. mexicanum* maintained substantial clonal diversity for all time periods and sites sampled. Most striking in these comparisons is the high proportion of infections that were multiclonal, even for the last year sampled (2005) after four years of low prevalence. At the sites with chronically very low prevalence, there was a significantly lower proportion of multiclonal infections, but still half of the infections contained more than one clone.

Overall genetic diversity (H) was high for all samples, including the low prevalence sites, including the low-prevalence sites where genetic diversity was not significantly different than for sites with much higher prevalence (Fig. 4.1). The single measure of clonal diversity that declined from high- to low-prevalence years was the number of alleles scored in the samples. Although these ‘lost’ alleles could have remained at their initially
low frequency (or even increased) and simply were missed by the sampling program, it more likely that these alleles either dropped much lower than their original frequency or were lost in the parasite metapopulation. During population bottlenecks, rare alleles are most likely to be lost first (Nei et al. 1975; Luikart et al. 1998), and most of the alleles not detected in later years had been rare alleles earlier.

Most striking in these comparisons is the high proportion of infections that were multiclonal, even for the last year sampled (2005) after 4 years of low prevalence. At the sites with chronically very low prevalence, there was a significantly lower proportion of multiclonal infections, but still half of the infections contained more than one clone. These results, and those for human malaria parasites (below), argue that mixed-clone infections are generally common for Plasmodium such that two to several genotypes of parasite will often co-exist in the same vertebrate host. These common genetically complex infections could lead to selection for competitive interactions among clones, or perhaps cooperation. Also, mixed-clone infections may be more harmful to the host (Read & Taylor 2001) so the observed genetic structure should play an important role in the evolution of virulence.

Changes in prevalence over time, and variation over space, is common for malaria parasites of both human and nonhuman vertebrate hosts (review in Schall & Marghoob 1985). However, such variation may not be sufficient to reduce genetic diversity; that is, a reduction in prevalence from 30% to 6% or even to 1% may not actually create a genetic bottleneck if the absolute number of hosts infected remains high. During a genetic bottleneck, as rare alleles are lost, the observed genetic diversity will appear
higher than expected under a specific model of mutation-drift equilibrium (Nei et al. 1975; Luikart et al. 1998). This excess in heterozygosity can thus test for any actual genetic bottleneck. Contradictory results emerged for two mutation models for \textit{P. mexicanum}. Under the IAM, a significant excess in heterozygosity was observed, even scoring only six loci, but no such excess was observed under the SMM (Table 4.4). A similar result was found for the human malaria parasite, \textit{P. falciparum}, for sites where a bottleneck was suspected (Machado et al. 2004). Simulations demonstrate that the predicted heterozygosity excess is difficult to detect under SMM, but becomes obvious when there is even a slight tendency for alleles to mutate via the IAM model (Luikart et al. 1998). Thus, the results are equivocal for the \textit{P. mexicanum} system, leaving an open question as to the real impact of reductions in prevalence for malaria parasites. In any case, genetic diversity remained high for the parasite metapopulation, and number of mixed infections were common, even for sites where prevalence has been low for many years.

Our methods in scoring clonal diversity followed those of Anderson et al. (2000) so that we can compare the results for \textit{P. mexicanum} with a world-wide survey of genetic diversity in \textit{P. falciparum} in humans (Fig. 4.1). The relationship between transmission intensity (estimated using prevalence) and both proportion of infections that were multiclonal and the overall clonal diversity (H) for \textit{P. falciparum} are nonlinear. Some areas with low transmission revealed low clonal diversity and fewer mixed infections, but other areas with similar transmission maintained high clonal diversity (Fig. 4.1). For \textit{P.}}
mexicanum, overall clonal diversity was high for all levels of prevalence, and mixed infections were less common only for the lowest prevalence sample (LowestYr = 2005).

The similarity of the results for a lizard malaria parasite and P. falciparum worldwide suggests that high clonal diversity of malaria parasites may be a general situation. This conflicts with Tibayrenc et al. (1991) who suggest that malaria parasites may maintain low levels of genetic diversity, even uniclonal at some sites (see Durand et al. 2003 for a similar result using microsatellite markers). Several features of Plasmodium biology would seem to favor a reduction in genetic diversity. Assortative mating within the vector between identical gametes would yield identical haploid cells for transmission to the next vertebrate host, although the degree of mating between identical gametes remains an open question for Plasmodium (Anderson et al. 2000b; Razakandrainibe et al. 2005). Clones could be lost during transmission between vertebrate and insect hosts, thus gradually reducing the genetic diversity in the metapopulation. Large scale bottlenecks could occur during periods of low transmission when effective population size declines (Price 1980). Despite the plausibility of these arguments, measures of genetic diversity for human malaria parasites using surface proteins revealed multiclonal infections are common, especially in areas with high transmission (Paul et al. 1995; Paul et al. 1998; Gilbert et al. 1998; Felger et al. 1999; Konaté et al. 1999; Babiker et al. 1999; Cui et al. 2003). Use of surface protein genes to probe the clonal diversity of Plasmodium infections may be questioned because of the very high mutation rate for the loci and selection for diversity to match the host immune challenge (Hughes & Hughes 1995; Anderson et al. 2000; Mascorro et al. 2005). Microsatellite loci have been proposed to
be better indicators of clonal diversity; studies using these markers also reveal substantial clonal diversity within and among infections for two species of human malaria parasite (Anderson et al. 2000; Ferreira et al. 2002; Durand et al. 2003; Bruce et al. 2006). We chose to use microsatellites in the study of *P. mexicanum* because of their more likely neutral nature (and surface protein genes have not been discovered for this parasite species).

The results of studies on human malaria parasites as well as our findings for *P. mexicanum* suggest an intriguing question. Why does overall genetic diversity remain high and multiclonal infections remain common even during long periods of reduced transmission and at sites with low parasite prevalence? At the Hopland sites with only 1% or less of lizards infected over a period of decades, an estimate of overall clonal diversity (expected heterozygosity) is as high as 0.91, and fully half of infections are multiclonal. Likewise, genetic diversity remains high in metapopulations of *P. falciparum* at some sites with low prevalence. Gemmill et al. (1997) proposed that sexual reproduction in parasites functions to generate genetic diversity in offspring to face a changing environment as hosts develop resistance to infection. The results for *Plasmodium* suggest there may be selection favoring genetic diversity in the parasite, perhaps as a result of the diversity of immune responses developed by hosts.
Acknowledgements

We thank numerous field assistants who collected lizards over the years at the Hopland site, and K. Kaufhold for assistance in the laboratory. The staff at the Hopland Research and Extension Center always provided a warm welcome and substantial logistical support. Tim Anderson and Bill Kilpatrick offered numerous suggestions that improved the study. E. M. Hart wrote the program for calculation of confidence intervals on expected heterozygosity. Funding was provided by the US NSF to JJS.
Literature Cited


Detecting recent reductions in the effective population size using allele frequency data. Journal of Heredity 90, 502-503.


Table 4.1. Number of clones (genotypes) of the malaria parasite, *Plasmodium mexicanum*, within individual infections of the vertebrate host, the western fence lizard *Sceloporus occidentalis*. Total clones is estimated as the maximum number of alleles detected for three microsatellite loci. Given is the number of infections, mean number of clones per infection, and percent of infections that were multiclonal. Four samples are compared, samples for a continuous period of years during which prevalence of the parasite was high (HiYrs), subsequent years when prevalence was low (LowYrs), and the most recent low prevalence year when prevalence was at its 28 year low (LowestYr). The last sample was from a set of sites where prevalence has been very low for the past 28 years (LowSites).

<table>
<thead>
<tr>
<th>N Clones</th>
<th>HiYrs</th>
<th>LowYrs</th>
<th>LowestYr</th>
<th>LowSites</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>11</td>
<td>9</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>42</td>
<td>29</td>
<td>24</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>21</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>3</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

| Mean Clones | 2.45 | 2.25 | 1.98 | 1.80 |
| N           | 101  | 64   | 41   | 40   |
| % Multiclonal | 88.1 | 82.8 | 78.0 | 50.0 |
Table 4.2. (Next page) Measures of clonal diversity for the malaria parasite *Plasmodium mexicanum* in its lizard host based on alleles for six microsatellite loci. Given are results for sites with relatively high prevalence of the parasite in lizard hosts at the study area for years with overall high (HiYrs) and low (LowYrs) prevalence, the last year with low prevalence (LowestYr), and for sites during the high prevalence years which consistently show low prevalence of the parasite (LowSites). Clonal diversity measures are number of alleles detected for each locus (# Alleles), range in number of a three base repeat for the microsatellite for each locus (Size Range in number of repeats), expected heterozygosity (H), effective population size (Ne), and proportion of the infections that were multiclonal (Multiclonal). Two measures are given, using all peaks on the pherograms that were > 1/3 the height of the highest peak (first value) and only using the highest peak (second value). For the estimate of percentage of infections that were multiclonal, all peaks > 50 fluorescent units on the pherograms were counted. Sample sizes are given in the first column as “total number of infections surveyed for that locus, total number of alleles counted among all infections”).
<table>
<thead>
<tr>
<th>Locus</th>
<th># Alleles</th>
<th>Size range</th>
<th>H</th>
<th>Ne</th>
<th>% Multiclonal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pmx306</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HiYrs (92, 131)</td>
<td>17 (17)</td>
<td>19 (19)</td>
<td>0.89 (0.86)</td>
<td>64.2 (39.3)</td>
<td>59.1</td>
</tr>
<tr>
<td>LowYrs (62, 88)</td>
<td>15 (14)</td>
<td>18 (18)</td>
<td>0.88 (0.87)</td>
<td>52.1 (49.2)</td>
<td>48.4</td>
</tr>
<tr>
<td>LowestYr (40, 51)</td>
<td>10 (9)</td>
<td>15 (15)</td>
<td>0.85 (0.85)</td>
<td>34.1 (34.1)</td>
<td>45.0</td>
</tr>
<tr>
<td>LowSites (31, 46)</td>
<td>14 (12)</td>
<td>13 (13)</td>
<td>0.89 (0.88)</td>
<td>64.2 (53.8)</td>
<td>45.0</td>
</tr>
<tr>
<td>Pmx732</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HiYrs (70, 89)</td>
<td>17 (14)</td>
<td>33 (32)</td>
<td>0.83 (0.78)</td>
<td>26.4 (15.4)</td>
<td>33.0</td>
</tr>
<tr>
<td>LowYrs (64, 88)</td>
<td>12 (11)</td>
<td>33 (33)</td>
<td>0.81 (0.76)</td>
<td>21.4 (12.7)</td>
<td>50.0</td>
</tr>
<tr>
<td>LowestYr (38, 45)</td>
<td>7 (7)</td>
<td>26 (26)</td>
<td>0.82 (0.70)</td>
<td>23.5 (7.9)</td>
<td>39.5</td>
</tr>
<tr>
<td>LowSites (24, 25)</td>
<td>11 (10)</td>
<td>22 (22)</td>
<td>0.92 (0.91)</td>
<td>122.0 (96.3)</td>
<td>12.5</td>
</tr>
<tr>
<td>Pmx747</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HiYrs (98, 155)</td>
<td>16 (14)</td>
<td>21 (21)</td>
<td>0.90 (0.89)</td>
<td>77.8 (64.2)</td>
<td>67.3</td>
</tr>
<tr>
<td>LowYrs (64, 76)</td>
<td>15 (13)</td>
<td>17 (17)</td>
<td>0.88 (0.85)</td>
<td>40.3 (34.2)</td>
<td>34.4</td>
</tr>
<tr>
<td>LowestYr (40, 61)</td>
<td>9 (7)</td>
<td>13 (13)</td>
<td>0.64 (0.51)</td>
<td>5.3 (2.5)</td>
<td>70.0</td>
</tr>
<tr>
<td>LowSites (34, 39)</td>
<td>9 (9)</td>
<td>18 (18)</td>
<td>0.78 (0.78)</td>
<td>15.4 (15.4)</td>
<td>32.0</td>
</tr>
<tr>
<td>Pmx328</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LowYrs (68, 79)</td>
<td>4 (3)</td>
<td>11 (10)</td>
<td>0.41 (0.38)</td>
<td>1.5 (1.2)</td>
<td>14.0</td>
</tr>
<tr>
<td>LowestYr (44, 45)</td>
<td>3 (3)</td>
<td>10 (10)</td>
<td>0.24 (0.24)</td>
<td>0.6 (0.6)</td>
<td>2.0</td>
</tr>
<tr>
<td>Pmx710</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LowYrs (63, 77)</td>
<td>15 (14)</td>
<td>22 (22)</td>
<td>0.88 (0.94)</td>
<td>217.5 (53.8)</td>
<td>21.0</td>
</tr>
<tr>
<td>LowestYr (23, 26)</td>
<td>8 (8)</td>
<td>11 (11)</td>
<td>0.87 (0.86)</td>
<td>42.9 (38.7)</td>
<td>13.0</td>
</tr>
<tr>
<td>Pmx839</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LowYrs (69, 87)</td>
<td>13 (13)</td>
<td>36 (36)</td>
<td>0.87 (0.87)</td>
<td>45.7 (45.7)</td>
<td>25.0</td>
</tr>
<tr>
<td>LowestYr (33, 39)</td>
<td>8 (8)</td>
<td>13 (13)</td>
<td>0.85 (0.85)</td>
<td>34.6 (33.3)</td>
<td>18.0</td>
</tr>
</tbody>
</table>
Table 4.3. Number of alleles that were present in infections of *Plasmodium mexicanum* in its lizard host during a period of years with high overall prevalence (25 – 32%, 1996 - 1998), but were missing in samples taken after prevalence dropped (6 – 12%, 2001 – 2005). Three loci are given with the initial frequency of the lost clones. Note that most alleles missing in later samples were initially rare in the parasite metapopulation.

**Frequency of alleles in 1996 – 1998 sample**

<table>
<thead>
<tr>
<th>Loci</th>
<th>&lt; 1 – 1%</th>
<th>2%</th>
<th>3%</th>
<th>4%</th>
<th>5%</th>
<th>6 – 11%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pmx306</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pmx732</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Pmx747</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.4. Number of microsatellite loci (total of six scored) revealing an excess in expected heterozygosity for *Plasmodium mexicanum* when compared to expected values under mutation-drift equilibrium (calculated with BOTTLENECK software). Two mutation models were employed, the infinite alleles model (IAM) in which any size allele can mutate to any other size, and the stepwise mutation model (SMM) in which any size allele can mutate only to an increase or decrease of one repeat unit. Two samples of genetic diversity of *Plasmodium mexicanum* are given. The first (LowYrs) was a period of four years of low infection prevalence that followed a long period of much higher prevalence, and the second (LowestYr) was the next year which had the lowest prevalence recorded over a 28-year period. Significance level is given under the Wilcoxon test for number of loci under expectation of an excess in expected heterozygosity.

<table>
<thead>
<tr>
<th>Sample</th>
<th>IAM N loci H excess</th>
<th>P</th>
<th>SMM N loci H excess</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>LowYrs</td>
<td>5</td>
<td>0.016</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td>LowestYr</td>
<td>5</td>
<td>0.039</td>
<td>2</td>
<td>0.945</td>
</tr>
</tbody>
</table>
Figure Captions

**Figure 4.1.** Measures of clonal diversity for *Plasmodium mexicanum* compared with those for *P. falciparum* (*P. falciparum* data extracted from Anderson et al. 2000). For both panels, four samples are compared for *P. mexicanum* that differed by prevalence. These are (from lowest to highest prevalence) LowSites, LowestYr, LowYrs, and HiYrs (see Methods). For each panel, confidence intervals for the *P. falciparum* data could not be calculated because only summary measures were available from the literature.

(A) A measure of overall clonal diversity (expected heterozygosity = H) for *Plasmodium falciparum* and *P. mexicanum*. Data for *P. falciparum* are indicated with closed points. Results for *P. mexicanum* are given as 95% confidence intervals for H to allow comparisons among loci, samples, and with the *P. falciparum* data. Confidence intervals are given for three loci for *P. mexicanum* (Pmx 306 heavy line, 732 dashed line, 747 light line, from left to right for each sample).

(B) Similar data for percent of infections that were multiclonal, with 95% confidence intervals for percents given in Table 4.1.
Figure 4.1

A

Expected Heterozygosity

B

% Infections Multi-Clonal

Prevalence (% Lizards Infected)
Chapter 5

Experimental test for premunition in a lizard malaria parasite

(Plasmodium mexicanum)

Anne M. Vardo, Kimberly D. Kaufhold, and Jos. J. Schall

Abstract

Premunition in Plasmodium is the prevention of superinfection by novel genotypes entering an already established infection in a vertebrate host. Evidence for premunition was sought for the lizard malaria parasite, P. mexicanum, in its natural host, the fence lizard Sceloporus occidentalis. Clonal diversity (= alleles for the haploid parasite) was determined using 3 microsatellite markers. Both naturally infected lizards (N = 25) and previously noninfected lizards (N = 78) were inoculated IP with blood from donor infections and followed over a 3 mo period. Compared to the success of clonal establishment in all the naive lizards (78/78 successful), clones entering pre-existing infections had a significant disadvantage (9/25 successful). The number of pre-existing clones (1-2 vs. 3-4) within recipient infections had no effect on the success of superinfection. Infections that excluded entering novel clones did not have higher initial asexual parasitemia, but had a higher initial density of gametocytes, suggesting they were older. Infections allowing superinfection experienced a higher final parasitemia.

Introduction

Parasites could reduce the cost of intraspecific competition within a host by excluding entry of additional conspecific parasite larvae (helminths) or genotypes/clones (protists).
This process may be an adaptive manipulation of the host's immune response, or simply an incidental byproduct of normal host immune function (Brown and Grenfell, 2001). The effect is best known in the concomitant immunity of *Schistosoma* spp. infections (Smithers and Terry, 1967; Brown and Grenfell, 2001), but also seems likely in other helminths such as filarial worms (MacDonald *et al.*, 2002), and species of the protists, *Leishmania* (Anderson *et al.*, 2005), *Toxoplasma* (Cesbron-Delauw *et al.*, 1989), and *Plasmodium* (Smith *et al.*, 1999).

In the case of *Plasmodium* spp., the blocking of superinfection by genotypes that are novel to a chronic infection is a more limited version of concomitant immunity and is termed premunition (Sergent and Parrot, 1935). Premunition has now been detected in an experimental study of the rodent parasite, *P. chabaudi*, in a mouse model system (de Roode *et al.*, 2005) such that clones entering a mouse first have a competitive advantage over those arriving later in the experimental manipulations. The experimental protocol introduced parasites via infected blood into the host's circulation, indicating *P. chabaudi* premunition is, at least in part, a result of immune system response against blood stages (an additional response could attack incoming sporozoites or the forms in the liver). Chronic infections of *P. relictum*, with very low-level parasitemia, are also refractory to challenge with a novel strain even years after establishment of the original experimental infection, suggesting premunition is occurring for avian malaria parasites (Atkinson *et al.*, 2001).

No studies have sought evidence for premunition in the malaria parasites of reptiles (approximately half the known *Plasmodium* species infect lizard hosts; Schall, 1996).
Until recently, studies on the genetic diversity of reptile malaria parasites have not been possible because of the lack of known variable genetic markers to score clonal identity. Here, we test for premunition in the lizard malaria parasite, *P. mexicanum*, using recently described variable microsatellite markers for this parasite (Schall and Vardo, 2007). Identifying these markers allowed us to introduce novel genotypes of parasites into established, natural infections of *P. mexicanum*.

**Methods**

We conducted the study at the University of California Hopland Research and Extension Center, near the town of Hopland in Mendocino County, California. Since 1978, a long-term study at the Hopland site has examined the biology of *P. mexicanum* and its hosts, the western fence lizard, *Sceloporus occidentalis*, and the sandfly vectors, *Lutzomyia vexator* and *L. stewarti* (Schall and Marghoob, 1995; Schall, 1996, 2002).

Lizards were collected by noosing, and a drop of blood was taken from each animal to make a thin blood smear for staining with Giemsa. Another drop of blood was dried on filter paper for later genetic studies. We scanned the blood smears and selected naturally infected lizards for use in the experiments. We determined parasitemia in these infections by counting the number of asexual cells (trophozoites and schizonts) and gametocytes in 1,000 erythrocytes, taking care to count cells and parasites from areas throughout the smear. The clonal diversity and identity of clones for all naturally infected lizards in the study were scored using 3 microsatellite markers (Pmx306, 732, and 747; Schall and Vardo, 2007). DNA from a dried blood sample was extracted using the DNeasy kit (Qiagen, Valencia, California), and the 3 loci amplified by PCR using 1
florescent-labeled primer for each locus. Primers and PCR conditions are given in Schall and Vardo (2007). Fragment size (alleles) were detected with an ABI 3130x1 Genetic Analyzer and scored using GeneMapper v3.7 software (ABI).

We collected male *S. occidentalis* not infected with *P. mexicanum* (N = 78) from areas of the field site where the parasite has been absent in the lizards throughout the long-term study (Schall and Marghoob, 1995; J. Schall, unpubl. obs.). Infections so weak that parasites are not seen during a six minute scan of a smear are rare at Hopland (Perkins *et al.*, 1998). However, we confirmed that these lizards were not infected by using a PCR-based method that can detect extremely low parasitemia infections (Vardo *et al.*, 2005). Each noninfected lizard received an inoculation of infected whole blood IP containing approximately 200 x 10³ parasites in 0.02 ml of blood/PBS (Osgood *et al.*, 2003). Five infected donor lizards chosen as a source of blood for these inoculations harbored 1 - 3 clones (alleles) of parasites (alleles varied among both loci and donors).

A second group of naturally infected lizards was chosen to be experimental recipients (N = 25) and donors (N = 3). The 3 donors for these inoculations contained infections with 1, 1, and 3 clones over all loci.

Donor lizards were assigned to experimental lizards depending upon multi-locus genotypes, with each donor having at least one unique allele compared to its recipient lizard. The experimental naturally infected lizards were inoculated in the same manner as the non-infected lizards, with approximately 200 x 10³ asexual parasites in 0.02 ml blood/PBS injected IP into each infected recipient. Differing alleles in donor and recipient lizards allowed us to determine if the challenging clones became established.
After inoculation, all recipient lizards were maintained in large outdoor cages, and fed daily with mealworms and crickets. Blood to make blood smears and dried blood dots for later genotyping were made every 10 days from toe clips. All collecting and experimental procedures followed a protocol approved by the University of Vermont Animal Care and Use Committee.

**Results**

All 78 noninfected lizards that were inoculated with infected blood became infected after 1 mo (determined by examination of blood smears). Donors with single-clone infections (at all loci) established infections in all recipients and most clones in multicleone donor blood were also successful in becoming established in the recipients (Fig. 5.1). In contrast, transmission was successful for only 9 of 25 natural infections that were challenged with entry of novel genetic clones. Thus, transmission was far more likely to be successful if the lizard was not already infected with *P. mexicanum* ($\chi^2 = 59.1, P < 0.0001$). Recipients already infected with 1 or 2 clones (across all 3 loci) were compared with those with 3 - 4 clones, found no difference in the ability of challenging novel clones to become infected (4/13 for recipients with 1 or 2 pre-existing clones; 5/12 for recipients with 3 - 4 clones; Fisher Exact Test, $P = 0.688$).

Although the results suggest that novel clones are indeed prevented from establishment in already infected lizards, the outcome could also be the result of donor effects. That is, some infections (donors) may simply be poor at becoming established in any lizard. Two donors containing a single parasite clone were successful at establishing the parasite in noninfected lizards (16/16). Success of donors with a single clone to
establish in already infected lizards was only 8/18 ($\chi^2 = 12.6, P = 0.0004$). For donors harboring 2 clones, establishment was successful in 15/15 noninfected lizards (with both clones becoming established in 12/15 recipients), but only 1/7 infected recipients, with only 1 of the clones becoming established in that single successful transfer ($\chi^2 = 9.673, P = 0.0019$). Thus, transmission was successful into noninfected lizards for all donor infections, and transmission was poor for all donors used to supply blood for the naturally infected recipients.

Comparison of parasitemia of pre-existing infections prior to introduction of the novel clones revealed no difference in numbers of asexual stages (trophozoites and schizonts) per 1,000 erythrocytes for successful and failed introductions ($U$-test, $P > 0.05$). However, the initial number of gametocytes and proportion of gametocytes among all parasites was greater for those recipients in which the introduced clones failed to become established than for the recipients that accepted the introduced clones. For introductions that failed, initial range of gametocyte density was 0 - 12/1,000 RBC and 17% - 75% gametocytes, and for introductions that succeeded, gametocyte density ranged from 0 to 5/1,000 RBC and 0% to 42% gametocytes ($U$-test for gametocyte parasitemia, $P = 0.0012$, and proportion of gametocytes, $P = 0.0002$). Final parasitemia was also lower for infections in which the novel clones failed to become established (Median = 5, range = 1 - 16/1,000 RBC) vs. those with successful establishment of the introduced clones (Median = 12, range = 5 - 244/1,000 RBC) ($U$-test, $P = 0.022$).
Discussion

The hypothesis of premunition in *Plasmodium* spp. infections proposes that established infections in the vertebrate host will hinder or exclude newly arriving novel genotypes, and genetically complex infections (multiclonal) would be more effective at hindering incoming genotypes than single-clone infections (Sergent and Parrot, 1935; Smith *et al.*, 1999; de Roode *et al.*, 2005).

We found that transmission by inoculation of whole infected blood into noninfected lizards was highly successful in establishing infection (all of 78 recipients became infected). When the donor infection was multiclonal, most clones became established in the recipients. However, already established infections were successful in limiting transmission of novel incoming clones, with approximately two-thirds resisting establishment of novel parasite genotype. Our results differ from previous proposals, though, because 1-2 clone infections were just as efficient in hindering the incoming clones as genetically more complex infections.

The natural infections we used had an unknown history prior to use in the experiment. The density of asexual-state parasites in the blood prior to the manipulation was not related to the success of incoming clones. However, infections with higher numbers and proportions of gametocytes were less likely to allow establishment of experimentally introduced novel clones. We suspect that these infections may have been older, because in both natural infections followed over time (Bromwich and Schall, 1986) and experimentally induced infections (Eisen and Schall, 2000) proportion of gametocytes in *P. mexicanum* infections generally increases with the duration of the infection. Older
infections may have had more time to induce a lasting immune response from the host, which may in turn limit the success of newly arrived parasites. At the end of the experiment the parasitemia of infections in which introduced clones became established was greater than the resistant infections similar to the increase in asexual replication noted for human malaria infections (Smith et al., 1999). It was not possible to determine if the overall higher rate of increase of the infections with established new clones was a result simply of the sum of all clones or if there were changes in the reproductive strategy of the genetically more complex infections.

The present study and that of de Roode et al. (2005) demonstrate that premunition in Plasmodium spp. is a result, at least in part, of processes taking place in the blood circulation, and some interference with the blood stages of the parasite. This effect most likely involves the host immune response, and if so, the P. mexicanum experiment provides rare evidence that reptiles mount an immune response to infection with malaria parasites. The only other evidence is the rapid destruction of host erythrocytes and appearance of immature red blood cells in circulation of infected lizards (Schall, 1990). Whether premunition observed for P. mexicanum represents an adaptive interference competition in which established parasites manipulate the host immune system is unknown, but an interesting possibility. Most natural infections we genotyped contained more than 1 clone of P. mexicanum (only 2 of the naturally infected lizards used as recipients contained a single genotype of parasites at all loci). If the effect seen in the experimental study occurs during natural transmission by the vector, this suggests that multi-clonal infections become established primarily by nearly simultaneous bites of
infected vectors or bites by vectors carrying multiple genotypes of parasite. Thus, premunition must play a role in the transmission biology of *P. mexicanum* and establishment of multi-clonal infections.

**Acknowledgments**

We thank the staff of the HREC, especially C. Vaughn, R. Kieffer, and R. Timm for logistical support and laboratory space. B. Blumberg assisted with the field work and maintaining captive lizards. The research was funded by a grant from the NSF to JJS and from the University of Vermont Helix program to KK.
Literature Cited


103


**Figure Captions**

**Figure 5.1.** Transmission success of genotypes (clones) of *Plasmodium mexicanum* resulting from experimental inoculation of whole blood from infected fence lizards, *Sceloporus occidentalis*, into previously noninfected lizards. Infections within the 5 donor lizards were genotyped for 3 microsatellite loci, and contained from 1 to 6 alleles (depending on infection and locus). The proportion of entering clones that became established (averaged over all loci) in 78 previously noninfected lizards is presented.
Figure 5.1

![Graph showing the proportion of clones successful for different numbers of clones entering the host.](image-url)
Chapter 6

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

Abstract

Cross-sectional surveys of both human malaria parasites (*Plasmodium falciparum*, *P. vivax* and *P. malariae*) and of the saurian malaria parasite *P. mexicanum* reveal that most infections are genetically complex. I explored the transmission dynamics of diverse *Plasmodium mexicanum* infections from its vertebrate host, *Sceloporus occidentalis*, to its sandfly vectors, *Lutzomyia vexator* and *L. stewarti*. I asked how the transmission biology of *P. mexicanum* can allow for such within-host diversity and how that diversity may alter transmission efficiency of infections. Sandflies were fed on experimentally induced infections harboring 1, 2 or 3+ clones for 24 hours and were dissected after 5 days to assess infectivity, oocyst burden, and genetic composition of the oocyst population. Multiclonal infections (3+ clones) had similar transmission success as infections harboring fewer clones. The majority of genotypes within infections were transmitted to the sandfly vector and contributed to the oocyst population. These results suggest that the transmission biology of *P. mexicanum* is conducive to maintaining genetic diversity within populations and multi-clonal infections can be established from a single bite. These findings shed light as to the common occurrence of multiclonal infections in this system, even in areas with consistently low transmission.
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). Within the blood meal, male and female gametocytes produce gametes that undergo sexual recombination to yield a diploid zygote. Meiosis and further development leads to the mobile ookinete that travels to the midgut wall where it develops into the oocyst. Repeated replication of parasite cells within the oocyst produce the next transmission stage, the sporozoites (Baton and Ranford-Cartwright 2005). All replicating stages in both hosts are haploid, an important feature of the life cycle for efforts to genotype infections. Transmission success of *Plasmodium* parasites 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), genotype of the parasite clones replicating in the vertebrate host (de Roode *et al.* 2005a and b), and vector genotype (Breier 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 known (but see de Roode *et al.* 2005a and b).

Cross-sectional surveys on human malaria parasite, *P. falciparum*, *P. vivax*, and *P. malariae*, (Anderson *et al.* 2000; Bruce *et al.* 2007; Cui *et al.* 2003; Imwong *et al.* 2006; Leclerc *et al.* 2002) and rare data on malaria parasites of nonhuman vertebrate hosts (Vardo and Schall 2007) reveal that infections commonly contain more than a single
genotype of parasite, and multiclonal infections are more common where transmission intensity is high. The occurrence of multiclonal infections suggests two questions on the role of genetic diversity for overall transmission biology and population genetics of the parasites. First, the small amount of blood taken during a vector feed may not include mature gametocytes of all clones within that infection, and there must be some likelihood that not all genotypes will successfully mate, form ookinetes, and complete sporogony. Thus there may be a bottleneck in transmission, with transfer of all genotypes being unlikely. This begs the question of how the common multiclonal infections become established; either transmission success is actually high for all clones in complex infections, with no bottleneck, or there are multiple bites by infected vectors carrying distinct genotypes of parasite. However, the first genotypes of *Plasmodium* becoming established appear to hinder later entry of novel genotypes (de Roode *et al.* 2005a; Vardo *et al.* 2007). Second, overall transmission success may be reduced for mixed-clone infections if clones compete to be established within the vector (de Roode *et al.* 2005b).

I sought to determine how the genetic diversity of clones in the vertebrate host’s blood influences transmission success of *P. mexicanum*, a parasite of fence lizards, *Sceloporus occidentalis*, and sandflies, *Lutzomyia vexator* and *L. stewarti*, in northern California, USA (Fialho and Schall 1995). This system has been under study for many years at a site in northern California (Schall 1996, 2002). The life cycle is similar to that of other *Plasmodium* species, with the exception of use of a sandfly vector rather than a mosquito. Recently characterized 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 - 85% depending on site). Experimental infections are readily established that vary in clonal diversity (Vardo-Zalik and Schall, 2008), and experimental feeds by sandflies produce oocysts that can be counted (Schall 2000; Fialho and Schall 1995) and genotyped (below). I established replicate experimental infections of *P. mexicanum* with known number of parasite genotypes in the natural vertebrate host species, and determined both overall transmission success and transmission success of individual clones into the sandfly vectors. 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.

**Methods**

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 from five annually sampled sites (sampled each year from 1978 to 2007), all within 1.2 km of 39° 00' 02" N, 123° 04' 39"W on the 2169 ha field station to serve as blood donors to initiate experimental infections. Blood was obtained from a toe clip to make blood smears (stained with Giemsa and examined at 1000 x) and dried and frozen 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 1000 erythrocytes). In early May, few infections presented high meront densities, so of the sample of 312 lizards, only seven suitable donors were
identified. *S. occidentalis* are small lizards, so only a small number of recipient infections could be initiated from a single donor.

The parasites from the donor infected lizards were genotyped for three microsatellite markers (Pmx 306, 747, and 732) using PCR primers and conditions presented in Schall and Vardo (2007) (Table 6.1). Labeled PCR product was run on the ABI Prism genetic analyzer and results analyzed using GeneMapper software (ABI). Two donors contained a single clone of parasites (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 represents the minimum number of clones present within an infection, and is proposed to be unbiased estimate of clonal diversity (Anderson *et al.* 2000; Ferreira *et al.* 2002; Bogreau *et al.* 2006; Bruce *et al.* 2007; Ferreira *et al.* 2007; Vardo and Schall 2007).

To create replicate recipient infections with known number of clones, 80 noninfected adult male lizards (snout-vent length >54 mm) were captured from two sites where malaria has been absent for at least 10 years (39° 02' 05" N, 123° 05' 49" N and 38° 59' 27" N, 123° 05' 27" W). Smears were examined at 1000x to view at least 10,000 erythrocytes, and a sensitive PCR-based protocol that detects extremely weak infections (as low as 1 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 ten recipients, were as follows: two single-clone groups, three two-clone groups, and three multi-clone groups (with three or more clones) (Table 6.2).

Infections were initiated following the protocol given in Vardo and Schall (2008). Recipient lizards were infected with blood from 1 or 2 different donor infections (Tables 6.1 and 6.2). Briefly, blood was taken from each donor lizard and quickly mixed with Phosphate Buffer Saline (PBS). Twenty microliters of the blood-PBS mixture (containing approximately 200 x 10^3 asexual parasites) was injected intraperitoneally into each recipient lizard. The number of parasites injected remained constant across all treatment groups regardless of clonality; for mixed-donor treatment groups, each donor contributed an equivalent number of parasites to the mixture, resulting in the final count of 200x10^3 asexual parasites in 20µl.

Lizards were housed by replicate in 8 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 bihourly from 1300-1900 hours.

Blood samples were taken one month after injection and also on the day of a feed to estimate gametocytemia (the number of mature male and female gametocytes within an infection) and gametocyte sex ratio (proportion microgametes, or males). Lizards in recipient group 1B were bled an additional 2 times before the one 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 2 drops of blood from each of the other treatment lizards were taken at the one-month bleed to account for this. Clonality within
each infection on the day of a feed was assessed at three polymorphic microsatellite loci (Pmx 306, 747 and 732) after the experimental period (Table 6.2).

Sandflies were captured nightly from July 8-August 26 from 2200-2400 hrs in funnel traps as described by Schall, 2000. 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 hrs in captivity, the sandflies (10-20 at a time) were allowed to feed on an infected lizard randomly chosen from a treatment group. 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 hr feeding period. Fed flies were removed and transferred to a separate jar in the incubator with a sucrose soaked cotton ball placed on top and changed daily. Any unfed flies were discarded.

After 5 days, flies were dissected in dH₂O, the species of sandfly recorded (Lutzomyia vexator or L. stewarti) and midguts examined for oocysts (Fialho and Schall 1995). I dissected flies on day 5 rather than day 9 as per Fialho and Schall (1995) to minimize bursting mature oocysts. If infected, the number of oocysts per midgut was counted and the gut stored in a 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 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 kimwipes, within a fume hood. After
24-48 hours, midguts were dry and their DNA extracted using the Qiagen DNeasy extraction protocol.

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. Preliminary trials found that repeated genotypes of infected lizard blood (from DNA extraction and PCR to fragment analysis by the DNA analyzer instrument) found that relative peak heights for mixed infections produced nearly identical relative peak heights (unpubl. data). Therefore, the relative peak heights allowed determination if the proportions of each clone within the lizard matched those in the vector. Because of the vagaries of the Prism instrument's function (ABI manual, 2004), we did not use the peak heights to estimate actual density of the parasites in lizard and vector, but only to determine if the density in the lizard matched that in the vector.

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+) as the main effect and replicate treatment groups (a, b, c) nested within clones. Gametocytemia on the day of the feed (# gametocytes observed in 1000 red blood cells) was $\log_{10}(n+1)$ 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.
Results

Sandfly feeds were conducted on 29 lizards (11 single-clone, 9 two-clone, and 9 multi-clone ‘3+’). One lizard in multi-clone treatment group 3A was fed upon twice, but was considered an independent treatment because 8 days had passed between vector feedings. Sandflies dissected were 131 *Lutzomyia vexator* and 30 *L. stewarti* (Table 6.3). 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 85 for single-clone infections, 56 for two-clone infections, and 34 for multi-clone infections (Table 6.3).

Gametocyte sex ratio for the 29 infections ranged from 0 - ~99% microgametocytes (mean 25%), and did not differ between clonal groups or replicate treatments (Kruskal-Wallis Test, $P = 0.43$ and 0.35). The distribution of gametocytemia (gametocytes per 1000 erythrocytes) also did not differ across treatment groups and replicates ($F_{7,21} = 0.25$, $P = 0.67$ and $P = 0.95$; Table 6.4).

Infectivity to the sandfly was not affected by clonality, but was influenced by replicate treatment ($\chi^2$ test, $P = 0.999$ and $P = 0.0003$ respectively). However, this result was largely influenced by one treatment group (3b) with only 3 sandflies, all of which did not become infected (Table 6.3). Removing this group from the analysis, the replicate effect remained ($\chi^2$ test, $P = 0.038$). Oocyst burden for the infected midguts ($N = 148$) ranged from 1 - 276 per midgut, with an overall mean of 56 per midgut. The number of oocysts did not differ among treatment groups (Fig. 6.1; Table 6.4), but replicate treatment group influenced oocyst burden ($F_{6,141} = 7.3$, $P = 0.82$ and $P = 0.0001$ respectively).
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). There was no association between gametocytemia and clonal diversity of the infections \( (F_{7,21} = 0.25, P = 0.67 \) for clonality and \( P = 0.95 \) for replicate). Fly species marginally influenced transmission success \( (F_{23,121} = 2.79 P = 0.043) \), with higher success for \( L. \) vexator, but sandfly species did not associate with differences in gametocytemia \( (F_{23,121} = 2.79 P = 0.259) \).

A random sample of 75 midguts was chosen to determine the transmission success of specific clone number and specific genotypes from lizard to the sandfly (15 single-clone, 29 two-clone, and 31 multi-clone infections). For these three groups, number of oocysts per midgut did not differ \( (F_{2,72} = 0.71, P = 0.49) \). 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 parasites developed \( (F_{6.2a} = 86\% \) and 74\% respectively). It is possible that some clones detected in the vector were not producing mature gametocytes at the time of the feed, but nonetheless, transmission success of all clones was efficient. Examination of the marker with greatest allelic diversity \( (Pmx306) \), 95\% of the two-allele infections passed both alleles to the vector, and 78\% of the multi-allele infections passed all alleles \( (F_{6.2b}) \).
Pherogram peak heights for two of the markers were compared for each lizard and infected sandfly to determine if the relative density of the parasites in the vertebrate was related to relative density in the vector. For Pmx306, relative peak heights were the same for 67% of the sandflies. For the sandflies displaying different relative peak heights compared to the source of their blood meal, all but one were infections with three alleles present. For marker Pmx747, 86% of the sandflies showed no difference in relative peak heights; those showing a switch were all two-allele infections.

**Discussion**

I present here the first examination of transmission success of genetically diverse malaria infections in a natural, non-human malaria system. Multiclonal infections are very common in malaria infections, including human pathogens *Plasmodium falciparum*, *P. malariae* and *P. vivax* and the saurian parasite *P. mexicanum* (Anderson *et al.* 2000; Bruce *et al.* 2007; Cui *et al.* 2003; Imwong *et al.* 2006; Leclerc *et al.* 2002; Vardo and Schall 2007). The ability for a clone to establish within an infection consisting of non-kin is only part of the obstacle for malaria parasite genotypes. Additionally, each genotype must successfully produce transmission stages at an appropriate sex ratio to ensure transmission and reproduction in the insect vector. In theory, competition between cohabitating genotypes may result in higher transmission stage densities or skewed sex ratios, as each genotype shifts to producing more gametocytes rather than asexual forms (Eisen and Schall 2000; Ferguson *et al.* 2003b; Taylor and Read 1998;
Taylor *et al.* 1997a). However, this may not result in greater transmission success if competition for establishment in the vector occurs (de Roode *et al.* 2005b).

The purpose of the present study was to examine the transmission efficiency of multiple genotypes within an infection and the relative transmission success of single and mixed-clone infections of the malaria parasite *Plasmodium mexicanum*. I aimed to determine if/how clonality within an infection alters transmission success, or if other factors, such as gametocytemia, genotype and/or vector species, were more important in dictating transmission proficiency. Additionally, I examined the genetic makeup of the oocyst population to assess the proportion of clones lost during the transmission cycle.

Experimental infections were initiated with 1-6 different parasite clones (albeit not the only clones found in nature) and wild-caught sandflies, *Lutzomyia vexator* and *L. stewarti*, were allowed to feed on infections for 24 hours. Transmission success was determined by infectivity to the sandfly and also by the number of oocysts produced. Of the 164 sandflies used in this study, only 3 could not be identified as either sandfly species and 8% did not become infected (Table 6.3).

I found that infections harboring 1 clone were just as successful infecting a sandfly and producing similar oocyst burdens as more diverse infections (Table 6.4). It is possible that the proportion of each genotype within a multi-clonal infection is not equally distributed among gametocytes, with only one clone dominating the transmission stage genotype. If this were true, one might expect similar oocyst burdens between single and multi-clonal infections. To examine the fate of each genotype present in an infection, a sample of infected midguts were genotyped and it was determined that most, if not all,
clones within mixed infections were present in the oocyst population. Therefore, each clone was producing gametocytes at the time of transmission. The multiclonal infections did not have higher oocyst burdens or abundance of transmission stages when compared with single-clone infections suggesting that competitive interactions between cohabitating genotypes may limit the transmission success of each clone.

A previous study on this system has shown that maximum gametocytemia within infections (not studied here) is higher for diverse infections (>1 clone) (Vardo-Zalik and Schall 2008). I did not observed such a correlation between gametocyte density and clonality in the present study, albeit different clones were utilized, which suggests that the relationship between clonality and gametocytemia is either genotype specific or weak during the growth phase of the infection.

In comparison of oocyst burdens between replicates within treatment groups, a replicate 1A produced significantly fewer oocysts than 4 other treatments. This, however, was attributed to the lower gametocytemia, although not significantly, in these infections, rather than clonality (Table 6.4). A genotype effect may explain the reduced gametocytemia observed in treatment group 1A. This group has allele “H” at locus 306; the only other group that has allele “H” is 3C, which also had lower gametocytemia and oocyst burdens (Tables 6.1, 6.2 and 6.4). It is unclear at this time whether a single allele difference could cause such a difference in this life history parameter. In a cross sectional examination of natural diversity within populations of *Sceloporus occidentalis*, we found that this allele is relatively common when compared to other alleles at this locus, with a frequency of ~ 17%.
It is not surprising that infection gametocytemia is positively correlated with transmission success; this has been a rather consistent finding in malaria transmission experiments on both human and nonhuman species. Schall examined transmission dynamics in *P. mexicanum* and found that up to a certain gametocytemia, an increase in oocyst burdens was found (Schall 2000). Any infection harboring more than this ‘threshold’ had similar oocyst burdens, not greater. This suggests that there may be a maximum number of gametocytes that can be picked up during a single feed or that a threshold for oocyst density within the sandfly vector may exist. If the latter is true, we could expect some fitness cost to infection for the vector, a subject still unresolved for most malaria parasites (Ferguson *et al.* 2003a; Ferguson and Read 2002; Ferguson *et al.* 2003b).

It is interesting to note the marginal significance sandfly species has on transmission (Table 6.2). *Lutzomyia vexator* is more abundant than *L. stewartii*, and is also a more competent vector, producing more oocysts. Vector competence can be important in modeling the transmission of disease, especially if vector species differ in their ability to become infected. For *Plasmodium falciparum*, two sympatric mosquito vectors do not appear to differ in their infectivity (Annan *et al.* 2007). Because our sample sizes for the two species were dramatically different, we believe that the difference observed is mainly an effect of unequal sample sizes.

These findings indicate that mixed-clone infections are as equally successful as single-clone infections in regards to transmission. Additionally, the reduction in parasite genetic diversity due to transmission bottlenecks appears to be minimal. This is an interesting
point, for it illustrates that competition between cohabitating clones does not reduce the genotypes present in the transmission stage population. I was unable to complete the transmission cycle to determine how many clones within the vector ultimately become established within the next infected host, but this preliminary experiment has shown that in mixed-clone infections, most, if not all genotypes are successful in transmission to the insect vector, reproduction and oocyst formation.

These results conflict with experiments utilizing the rodent malaria parasite *P. chabaudi* in a laboratory model. For *P. chabaudi*, mixed infections (often harboring 2-3 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 and Read 1998; Taylor *et al.* 1997a, 1997b). The parasite clones utilized in those experiments each express different virulence and growth phenotypes, something I have not been able to clearly address in our system. However, I did not observe a strong genotype effect in this or a previous study (Vardo-Zalik and Schall 2008).

The results from the present study highlight the importance of understanding transmission dynamics in malaria parasites for population genetic purposes. The ability for multi-clonal infections to transmit the majority of their genotypes to the insect vector is a piece of the puzzle to understanding how parasite diversity is maintained within and among populations. Further studies that follow transmission bottlenecks from the vector to the vertebrate host are warranted.
Acknowledgements

I thank the staff of the Hopland Research and Extension Center for offering their usual warm welcome and logistical support, especially C. Vaughn, R. Keefer, and R. Timm. S. Reece and M. Robinson assisted in catching and caring for lizards. N. Zalik assisted in lizard and vector capture, husbandry and feed experiments. L. Stevens helped with statistical analyses. Jos. J. Schall offered suggestions on an earlier version of the manuscript and assisted with field methodology. This research was funded by grants from the USA National Science Foundation and the Vermont Genetics Network to JJS.
Literature Cited


Medicine and Hygiene 90:621-624.


Table 6.1. Seven donor infections and their respective number of clones. Alleles and clonality were assessed via three microsatellite loci (Pmx 306, 747, and 732; Schall and Vardo 2007). Presented here is the minimum number of clones within an (estimated via the microsatellite locus with the most alleles for that infection). 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)

<table>
<thead>
<tr>
<th>Donor</th>
<th># Clones</th>
<th>Pmx306</th>
<th>Pmx747</th>
<th>Pmx732</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>H</td>
<td>4</td>
<td>c</td>
</tr>
<tr>
<td>II</td>
<td>1</td>
<td>G</td>
<td>4</td>
<td>c</td>
</tr>
<tr>
<td>III</td>
<td>2</td>
<td>A C</td>
<td>4</td>
<td>c f</td>
</tr>
<tr>
<td>IV</td>
<td>2</td>
<td>D F</td>
<td>6</td>
<td>d</td>
</tr>
<tr>
<td>V</td>
<td>2</td>
<td>D</td>
<td>4 5</td>
<td>a</td>
</tr>
<tr>
<td>VI</td>
<td>4</td>
<td>A D G</td>
<td>2 4</td>
<td>b c f</td>
</tr>
<tr>
<td>VII</td>
<td>4</td>
<td>B D E H</td>
<td>1 3</td>
<td>e f a</td>
</tr>
</tbody>
</table>
Table 6.2. Eight treatment groups represented by clonal group (1-3) and letter (a-b; replicate treatment). All groups have 10 individuals. Alleles and clonality were ased via three microsatellite loci (Pmx 306, 747, and 732; Schall and Vardo 2007). Presented here is the minimum number of clones within an (estimated via the microsatellite locus with the most alleles for that infection).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Donor(s)</th>
<th># clones</th>
<th>PMX306</th>
<th>PMX747</th>
<th>PMX732</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>I</td>
<td>1</td>
<td>H</td>
<td>4</td>
<td>c</td>
</tr>
<tr>
<td>1b</td>
<td>II</td>
<td>1</td>
<td>G</td>
<td>4</td>
<td>c</td>
</tr>
<tr>
<td>2a</td>
<td>III</td>
<td>2</td>
<td>A C</td>
<td>4</td>
<td>c e</td>
</tr>
<tr>
<td>2b</td>
<td>IV</td>
<td>2</td>
<td>D F</td>
<td>6</td>
<td>d</td>
</tr>
<tr>
<td>2c</td>
<td>V</td>
<td>2</td>
<td>D</td>
<td>4 5</td>
<td>a</td>
</tr>
<tr>
<td>3a</td>
<td>VI</td>
<td>4</td>
<td>A D G</td>
<td>2 4</td>
<td>b c f</td>
</tr>
<tr>
<td>3b</td>
<td>VI + VII</td>
<td>6</td>
<td>A D G B E H</td>
<td>2 4 1 3</td>
<td>b c f e a</td>
</tr>
<tr>
<td>3c</td>
<td>II + VII</td>
<td>5</td>
<td>B D E H G</td>
<td>1 3 4</td>
<td>c e f a</td>
</tr>
</tbody>
</table>

Table 6.3. The number and species of sandflies that fed on lizards from each treatment, along with the proportion of the flies that became infected for each replicate. The three ‘unknown’ sandflies were not included in the analyses because we could not properly assign them to one of the two species.

<table>
<thead>
<tr>
<th>Treatment</th>
<th># Sandflies</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L. vexator</td>
<td>L. stewarti</td>
<td>Unknown</td>
<td># Flies Not Infected</td>
<td>Prop. Flies Infected</td>
</tr>
<tr>
<td>1a</td>
<td>32</td>
<td>9</td>
<td>0</td>
<td>5</td>
<td>0.80</td>
</tr>
<tr>
<td>1b</td>
<td>19</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>2a</td>
<td>14</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>0.89</td>
</tr>
<tr>
<td>2b</td>
<td>12</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>2c</td>
<td>21</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>1.00</td>
</tr>
<tr>
<td>3a</td>
<td>28</td>
<td>6</td>
<td>0</td>
<td>3</td>
<td>0.92</td>
</tr>
<tr>
<td>3b</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0.00</td>
</tr>
<tr>
<td>3c</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Table 6.4. Summary data for 7 treatment groups. The alleles present at each of the three-microsatellite loci are presented, with the lowest letter/number representing the smallest allele. Presented here are the back transformed values for both gametocytemia and oocyst burden. For sex ratio of gametocytes, the median percent of microgametes (males) observed is given.

**Alleles present on day of feed**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pmx 306</th>
<th>Pmx 747</th>
<th>Pmx 732</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>H</td>
<td>4</td>
<td>c</td>
</tr>
<tr>
<td>1B</td>
<td>G</td>
<td>4</td>
<td>c</td>
</tr>
<tr>
<td>2A</td>
<td>A C</td>
<td>4</td>
<td>c e</td>
</tr>
<tr>
<td>2B</td>
<td>D F</td>
<td>6</td>
<td>d</td>
</tr>
<tr>
<td>2C</td>
<td>D</td>
<td>4 5</td>
<td>a</td>
</tr>
<tr>
<td>3A</td>
<td>A D G</td>
<td>2 4</td>
<td>b c f</td>
</tr>
<tr>
<td>3C</td>
<td>B D E H G</td>
<td>1 3 4 c e f a</td>
<td></td>
</tr>
</tbody>
</table>

**Gametocytemia**

<table>
<thead>
<tr>
<th>Treatment</th>
<th># infections</th>
<th>Mean (st. dev)</th>
<th>Range</th>
<th>Sex ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>7</td>
<td>7.7 (7.30)</td>
<td>2-21</td>
<td>0.1</td>
</tr>
<tr>
<td>1B</td>
<td>4</td>
<td>9.8 (5.7)</td>
<td>4-16</td>
<td>0.34</td>
</tr>
<tr>
<td>2A</td>
<td>3</td>
<td>13.7 (17.7)</td>
<td>2-34</td>
<td>0</td>
</tr>
<tr>
<td>2B</td>
<td>2</td>
<td>8.5 (9.2)</td>
<td>2-15</td>
<td>0.17</td>
</tr>
<tr>
<td>2C</td>
<td>4</td>
<td>13.5 (18.4)</td>
<td>2-41</td>
<td>0.43</td>
</tr>
<tr>
<td>3A</td>
<td>7</td>
<td>11.1 (11.0)</td>
<td>4-34</td>
<td>0.25</td>
</tr>
<tr>
<td>3C</td>
<td>1</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

**Oocyst burden**

<table>
<thead>
<tr>
<th>Treatment</th>
<th># midguts</th>
<th>Mean (st. dev)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>36</td>
<td>7.7 (4.5)</td>
<td>3-16.3</td>
</tr>
<tr>
<td>1B</td>
<td>22</td>
<td>10.6 (5.5)</td>
<td>3.8-19.5</td>
</tr>
<tr>
<td>2A</td>
<td>16</td>
<td>9.3 (3.9)</td>
<td>3-16.3</td>
</tr>
<tr>
<td>2B</td>
<td>16</td>
<td>8.9 (4.5)</td>
<td>3-15.7</td>
</tr>
<tr>
<td>2C</td>
<td>24</td>
<td>10.9 (3.0)</td>
<td>3.8-17.9</td>
</tr>
<tr>
<td>3A</td>
<td>31</td>
<td>9.6 (5.6)</td>
<td>3.8-18</td>
</tr>
<tr>
<td>3C</td>
<td>3</td>
<td>6.7 (8.1)</td>
<td>3-11.5</td>
</tr>
</tbody>
</table>
Figure Captions

**Figure 6.1.** Transmission success of 1, 2 and 3+ clone infections measured by # oocyst formed by day 5 post-feed. The mean number of oocysts per treatment is given; transmission success was not significantly different between clonal groups.

**Figure 6.2.** Transmission success of multi-clone infections from the lizard to the sandfly vector. Seventy-five midguts were analyzed at 2-3 microsatellite makers. (a) Success of multiple clones transmitted to the sandfly vector over 2-3 markers. (b) Success of individual alleles at locus Pmx 306 in transmitting to the vector. In both cases, the majority of infections passed all alleles/clones on.
Figure 6.1

- $X_1 = 52.3$
- $X_2 = 50.8$
- $X_{3+} = 69.6$
Figure 6.2a

Proportion of Clones Transmitted

# Clones in lizard

Figure 6.2b

Proportion Clones Transmitted

# Clones in Lizard: Locus Pmx 306
Chapter 7

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

Anne M. Vardo-Zalik and Jos. J. Schall

Abstract

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

**Introduction**

Microparasites (viruses, bacteria, and protists) replicate within an individual host as either a single genetically uniform clone, or as a complex mixture of clones (Anderson and May 1979; Read and Taylor 2001). Many theoretical studies, both verbal and mathematical, reason that the degree of clonal diversity within infections will play a central role in the ecology of microparasites, and ultimately of their hosts (May and Nowak 1995; Van Baalen and Sabelis 1995; Frank 1996; Read and Taylor 2000, 2001; Schall 2002). One common view is that high clonal diversity will be associated with a more rapid rate of parasite replication and greater final parasite loads, leading to an increase in pathology suffered by the host (Bull 1995; May and Nowak 1995). If this is true, any shifts in the phenotype of infections associated with clonal diversity may well influence the parasite's transmission success and prevalence in the host, with cascading effects on host populations (Taylor et al. 1997; Eisen and Schall 2000; Schall 2000).

Despite the likely significance of clonal diversity for the biology of parasites, including important pathogens of humans and wildlife species, data that relate the number of genotypes within an infection to the parasite's infection dynamics are surprisingly scant (Read and Taylor 2001). We approached this issue with an experimental study that manipulated the number of coexisting clones within infections of the lizard malaria parasite, *Plasmodium mexicanum*, in its natural vertebrate host, the western fence lizard,
Sceloporus occidentalis (Schall 1996). Our goal was to evaluate several competing views on how clonal diversity may influence the dynamics of microparasite infections.

Malaria parasites (Plasmodium) cycle between two hosts, a vertebrate (squamate reptile, bird, or mammal), and an insect vector (mosquito or sandfly) (Valkiunas, 2005). Within the vertebrate host, malaria parasites undergo bouts of asexual replication of haploid cells (meronts), and the proportion of blood cells infected (parasitemia) increases over time to some maximal level, which may hold constant or decline as the immune system becomes active. A fraction of the parasites develops into gametocytes, the nonreproducing cells that are transmitted to the biting insect vector when it takes a blood meal (Eisen and Schall 2000). The replication rate of the asexually replicating parasite cells, timing of the shift to production of gametocytes, and greatest meront and gametocyte parasitemia all vary substantially among natural infections of every Plasmodium species studied to date (Eisen and Schall 2000). For example, maximal parasitemia of P. mexicanum infections varies from << 1% to 90% of erythrocytes infected (Bromwich and Schall 1986; Eisen 2000). There is also variation in the clonal diversity among Plasmodium infections. Many studies on human malaria parasites (e. g. Anderson et al. 2000; Imwong 2006; Bruce et al. 2007), and rare data on the Plasmodium of nonhuman vertebrates (P. mexicanum; Vardo and Schall 2007), document that infections within the vertebrate may harbor one to many parasite clones. For P. mexicanum, mixed-clonal infections surveyed at several sites varied from 50% to 88% of infections sampled (Vardo and Schall 2007). Thus, malaria parasites provide excellent models to study the relationship between the dynamics of infection and clonal diversity.
Clonal diversity could influence the course of *Plasmodium* infections in several ways. In single-clone infections, the optimal phenotype for the clone would be a relatively simple strategy to balance loss of parasite cells when infected blood cells are removed from circulation, with production of gametocytes for transmission, and to maintain the host for maximal duration of the infection. That is, the course of infection would follow some optimal path to insure maximal possible transmission (Eisen and Schall 2000; Schall 2002). But in a mixed-clone infection, several outcomes are possible. First, competition for limiting resources within the host could hold overall parasite growth rate and maximal parasitemia constant for both single or mixed-clone infections. Each clone would therefore suffer reduction in density caused by competition (de Roode *et al.* 2003). Second, mixed-clone infections could be more productive, with higher replication rates and higher maximal parasitemia, either because the final parasitemia is a sum over all clones (no interaction) (Schall 2000; de Roode *et al.* 2003), clones increase their rate of replication (competition for transmission), or more readily elude the immune system (Taylor *et al.* 1998; Eisen & Schall 2000; Ferguson *et al.* 2003). Third, competition among clones within complex infections may be severe and reduce the final overall parasitemia, especially if a mixture of parasite genotypes elicits a stronger immune response by the host (de Roode *et al.* 2005). Last, competition among clones for transmission to the vector could lead to earlier production of gametocytes in mixed-clone infections.

Evidence for the role clonal diversity plays on the infection dynamics of malaria parasites emerges from surveys of natural human malaria infections and experimental
studies of laboratory model systems. Studies on human malaria cannot experimentally alter the clonal diversity of infections, and must by necessity be only glimpses into events within each infection (Ferreira et al. 2002). A more controlled picture comes from manipulative experiments using laboratory model systems, such as the African rodent malaria parasite *P. chabaudi* in laboratory mice (Mackinnon and Read 2004). Laboratory model systems have an important shortcoming, however, because they do not examine natural parasite-host associations that have coevolved in real ecological settings. We examined the influence of clonal diversity on *P. mexicanum* by determining the replication rate of meronts, maximum meront parasitemia, timing of first production of gametocytes, rate of gametocyte increase, and maximum gametocyte parasitemia to compare results for single-clone or mixed-clone infections. All of these traits vary substantially among natural infections of *P. mexicanum* (Bromwich and Schall 1986; Eisen 2000).

### Methods

**Study site and collection of lizards**

The study was conducted at the University of California Hopland Research and Extension Center (here: "Hopland"), a 2,160 ha track of oak-woodland in southern Mendocino County, California. Hopland has been the focus of a long-term study from 1978 - 2007 that has examined the biology of *P. mexicanum* in its hosts, the western fence lizard, *Sceloporus occidentalis*, and two psychodid sandflies, *Lutzomyia vexator*, and *L. stewartii*, including the effects of infection on both vertebrate and insect hosts.
(Fialho and Schall 1995; Schall 1996, 2002). Prevalence of P. mexicanum has varied both among local sites at Hopland and among years (Schall and Marghoob 1995; Schall 1996, and subsequent data). The parasite has been absent from some sites since 1978, whereas at other sites, prevalence has ranged from a low of ~6% in 2005 to a high of ~35% in some earlier years.

During May, 2005, experimental infections were initiated in 69 noninfected adult male fence lizards (snout to vent length 65 - 75 mm) that were collected from two sites at Hopland where P. mexicanum was absent in the lizards since 1978 (39° 02' 05" N, 123° 05' 49" W and 38° 59' 27" N, 123° 05' 27" W) (Schall and Marghoob 1995 and subsequent unpublished data). To insure that the recipient lizards were noninfected, blood smears (stained with Giemsa) were scanned at 1000 x to examine > 10,000 erythrocytes. Also, a PCR-based protocol that detects extremely weak infections that are not patent under the microscope (as weak as 1 parasite cell per millions of erythrocytes) confirmed the noninfected status of these lizards (Perkins et al. 1998, Vardo et al. 2005). A control group of 19 noninfected male lizards from the collecting sites was kept for the study's duration, and all remained noninfected by both microscopy and PCR analysis. Infected donor lizards were identified from a sample of 484 lizards collected from sites where malaria has been most common over the past three decades (all within 1.2 km of 39° 00' 02" N, 123° 04' 39" W). Infections with high parasitemia of replicating meronts (> 25 parasites per 1000 erythrocytes) were rare during the sample period because prevalence was low in 2005 and such infections are always uncommon early in the warm season (Bromwich and Schall 1986). Only five lizards were suitable to serve as donors
of infected blood. Two measures of lizard health were taken for each animal prior to its introduction into an experiment: proportion of erythrocytes that were immature (Schall 2002) and tail condition (normal, broken, regenerated). All procedures followed an animal care and use protocol approved by the University of Vermont.

*Genotyping infections*

Blood samples from the donor and experimentally infected lizards were taken from a toe clip and stored frozen as dried dots made on filter paper (Vardo and Schall 2007). DNA was extracted from dried dots with the Qiagen DNeasy kit. Three microsatellite loci were amplified, Pmx306, Pmx732, and Pmx747 using primers and PCR conditions given in Schall and Vardo (2007), and Ready-to-Go PCR beads (GE Health Care). Samples were processed on an ABI 310 genetic analyzer, and data analyzed with GeneMapper 3.5 software (ABI). Because the parasite stages in the vertebrate host are haploid, each peak on the GeneMapper pherogram represents a single-clone of parasites (Anderson *et al.* 2000; Vardo and Schall 2007). An infection with a single allele at each locus was scored as a "single clone" infection. Previous experience (Vardo and Schall 2007) found that increasing the number of loci to six did not find additional clones in such infections. An infection with two alleles at one locus and one at the other two loci was scored as a "two clone" infection. Infections with two or more alleles at > 1 locus were problematic. To score clonal diversity in these infections, we calculated the minimum and maximum number of clones possible, based on the three loci. The minimum number of clones within an infection is the maximum number of alleles seen
for any of the loci (Anderson et al. 2000; Vardo and Schall 2007). The maximum present is the product of the number of alleles seen across the three loci. The minimum value may be an underestimate, and the maximum value is most likely an overestimate. We therefore report clonal numbers as the possible range for these infections. Blood samples taken at 30 days and 80 days post injection for each recipient lizard were processed to score the number of clones that had become established. This protocol found that three of the lizards lost a clone. In each case, they had received two clones, but only one clone became established, so these were moved to a "one clone" treatment group (below).

**Experimental infections**

Natural single-clone infections are uncommon at the site (~15% of infections; Vardo and Schall 2007), and lizards used to provide infected blood are small (~10 gm). Therefore, equal (and large) sample sizes of recipient infections with well-characterized parasite genotypes were not possible, a shortcoming of such field studies on a natural parasite system. Our goal was to generate infections within the normal range of clonal numbers seen in natural infections, and thus the situation actually experienced by the parasite. Five donor lizards were selected, two with a single clone (Donors I and II, each with a different genotype), one with two clones (Donor III with two genotypes not present in Donors I and II), and two with 2 - 8 possible clones (Donors IV and V) (criteria stated above), each harboring at least 1 unique genotype. Thus, within all five donor infections, 7-15 clones were available for study. Table 7.1 lists the donors, allele identification, and number of clones for donor lizards.
Parasitemia was determined for each donor by counting meronts in 1000 erythrocytes, and erythrocyte density measured with a Hausser Levy counting chamber (Osgood and Schall 2003). Experimental infections were initiated via intraperitoneal injection of donor blood. Briefly, blood taken from a donor lizard was mixed with phosphate buffered saline, and the sample diluted to contain $2 \times 10^5$ meronts in 20 mL of blood/saline mix. Blood from multiple donors was mixed to insure that the same number of parasite cells from each donor was injected into recipient lizards.

The nine treatment groups, with information on specific alleles present and sample sizes, are given in Table 7.2, and are named based on the number of clones present. The recipient lizards and control lizards were housed in 12 large outdoor vector-proof cages, and fed each day to satiation with mealworms and crickets. Each treatment group was housed in separate cages (to eliminate even the slightest chance that the parasite could be mechanically transmitted across treatment groups; Schall and Smith 2006). The cages were hung from an overhead wire, and were randomly moved each week in the 5 x 2 m array. Lizards were individually identified by the pattern of toe clips (two toes clipped on each lizard).

**Infection traits**

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

The course of a *P. mexicanum* infection typically takes 60 - 80 days to reach maximal parasitemia and maximal production of gametocytes, and thus samples taken every 10 days give a good indication of events during the course of infection (Eisen and Schall 2000; Osgood and Schall 2003). The slides were examined to count the number of parasites seen in 1000 erythrocytes from all areas of the smear, scoring number of meronts and gametocytes. Parasite numbers are reported as parasitemia, the proportion of erythrocytes infected, rather than parasite numbers per volume of blood because infection does not affect blood cell density (Schall 2002). Infections followed two patterns: parasitemia rose to a peak, then dropped (often to reach some stable lower point), or rose to a high stable level. Data for each infection were examined to score the dates on which the infection reached its highest meront and gametocyte parasitemia. For infections that maintained a high constant parasitemia, the first date was chosen as one score of peak parasitemia. However, infections that reached this peak, then dropped, would produce fewer parasites over time than those that maintained their highest parasitemia for weeks. Therefore, for lizards that lived the full 80 days of the experiment (66 of 69) total parasites seen in 1000 erythrocytes was summed for all samples as a second measure of maximal parasite numbers produced.

The rate of increase of both meront and gametocyte parasitemia was calculated as the highest parasitemia (first date for those reaching a stable point) minus the parasitemia when parasites were first observed on the smears divided by the number of days between
these two samples. First production of gametocytes was scored in two ways, the days from injection of infected blood into the lizard to appearance of gametocytes, and the days from first appearance of meronts in the blood to the first appearance of gametocytes.

Recipient lizards were scored for the number of clones establishing using blood samples taken at 30 days and 80 days post injection. As with the naturally infected lizards, three microsatellite loci were amplified and the number of clones within each infection compared to the predicted based on the injected donor blood. Three of the two-clone treatment infections had lost a clone. In each case, they had received two clones, but lost only one became established, so these were moved to a "one clone" treatment (Table 7.2).

Analysis

All analyses for treatment vs. infection trait were performed with a nested ANOVA design. Five main treatments were 1 clone (3 treatments), 2 clones (1 treatment), 2-8 clones (2 treatments: 2-4 and 2-8 clones), 4-32 clones (2 treatments), and 6-60 clones (1 treatment). Thus, data were nested as individual infection within replicate treatment group, within main treatment groups. Infection traits were combined in a Principal Component analysis to correct for possible correlation among traits. Data for all infection traits were not normally distributed, and so for ANOVA and t-tests they were log_{10} transformed. Transformed data were not significantly different from normal in distribution (Shapiro-Wilk goodness of fit test, Bartlett test, P > 0.05). Some data could not be rendered normal by any transformation (first production of gametocytes, site of
capture, proportion immature red blood cells, tail status and Principal Component scores) and thus nonparametric Kruskal-Wallis and Mann-Whitney tests were performed.

**Results**

*Growth rates, maximal parasitemia, and first production of gametocytes*

Data for the Treatment groups are given in Table 7.3 and Fig. 7.1. Results of the nested ANOVA tests are given in Table 7.4, showing main treatment effects and effect of replicate treatments nested within those main treatments. Meront growth rate, maximal meront parasitemia, and sum of meront parasitemia over all sample periods did not differ by main treatment or replicate treatments nested within the main treatment groups (all $P > 0.05$) (Table 7.4). Grouping data as 1 vs. > 1 clone infections, also found no difference in meront growth rate and maximal parasitemia ($P > 0.05$). Similarly, rate of increase of gametocytes, maximal gametocyte parasitemia, and sum of gametocytes over all samples did not differ among main treatment groups ($P > 0.05$). Combining all mixed-clone infections, however, gametocyte growth rate and maximal gametocyte parasitemia were higher for the mixed-clone infections ($P= 0.005$ and 0.007 respectively).

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

We performed two additional nested ANOVA tests to further compare the effect of increased clonality within infections. First, we compared treatments 1A and 1B with treatment 2, which was initiated with the same two donors (Table 7.2). Treatment 2 had similar maximum meront and gametocyte parasitemia ($F_{1,20} = 1.91, 3.49; P = 0.18$ and $P = 0.08$) but had higher rates of meront and gametocyte production ($F_{1,20} = 4.2, 6.28; P = 0.05$ and $P = 0.02$). Next, we analyzed treatments 2-4 and 2-8 with treatments 4-32A and B. We found no difference in meront or gametocyte parasitemia ($F_{1,32} = 0.11, 0.0002; P = 0.74$ and 0.99) or their rates of production ($F_{1,32} = 0.11, 0.12; P = 0.74$ and 0.73).

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

Examination of the raw, nontransformed data revealed a difference in variance for single-clone and mixed-clone infections for meront and gametocyte rates of increase and for their maximal parasitemias (Bartlett tests, all $P < 0.001$) (Fig. 7.1). For the infection traits shown in Fig. 7.1, we defined "High" infections as those with values $> x + 1 \text{ sd}$ for the total data set of 69 infections, and "Low" infections as those values $< x + 1 \text{ sd}$. None of the single-clone infections fell in the High group, but 20 of the 52 mixed-clone infections (39%) were High for one or more of the infection traits. We asked if the High infections for one trait were also the High infections for other traits. Only 4 fell into the High class for all traits, whereas 9 infections were High for only one trait, 3 for two traits and 4 for three traits. Thus, High infections were not the same infections for all four reproductive traits of infections. Clonal diversity may have no role in the 20/69 (29%) total infections being High for at least one infection trait; that is, by chance the 17 single-clone infections may have been drawn from the 71% of overall infections that had lower productivity. This has a low likelihood (the probability of randomly drawing a sample of 17 Low infections from a population that contains 71% Low infections = $0.71^{17} = 0.003$).

It is possible that specific alleles at one or more loci are associated with higher growth rates or higher parasitemia and mixed-clone infections could be more likely to contain these genotypes by chance. Ideally, each clone in the mixed-clone infections would be examined in replicate single-clone infections, but this was not possible because of the small number of suitable donor infections available in the study. We therefore examined the data in several ways to detect any hint of a clone or allele effect. First, the ideal
situation was available for treatment group 2 which contained the two clones found in two of the single-clone groups (groups 1a and 1b). Treatment group 2 included infections that were High in three of four life history traits, but the donor infections (1a and 1b) did not produce any exceptionally high rates of increase or maximal parasitemia. Second, for each of the three microsatellite loci, most alleles were found in both the High and Low infections. For example, for asexual growth rate, 6 of 6 Pmx306 alleles were seen in both the High and Low infections, 4 of 6 alleles for Pmx732 were seen in both groups, and 4 of 5 for Pmx747. Very similar results were seen for the other traits. Third, the tallest peak on the pherograms for each of the mixed-clone infections gives an indication of the predominant clone in an infection (in a few infections, a second or third peak was within 5% of the fluorescent units, so these were also scored as predominant peaks). Four of the six alleles for locus Pmx306 presented the highest peaks for both the Low and High infections for at least one infection trait. Similar results were seen for locus Pmx747 (2/5 alleles) and Pmx732 (3/6 alleles). Last, the same multi-allele combinations for each locus were found in both the High and Low infections. For meront growth rate, 6 pairs of infections had the same combination of alleles, for gametocyte growth rate, 7 pairs had the same combination, for maximum asexual parasites, 5 pairs matched, and for maximum gametocytes, 5 pairs matched. Thus, no hint is seen for any allele-specific effect on the infection traits.

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

**Discussion**

Every study of natural *Plasmodium* infections has found that mixed-clone infections are common, including those of the human malaria parasites *P. falciparum* (Anderson et al. 2000), *P. vivax* (Imwong et al. 2006), and *P. malariae* (Bruce *et al.* 2007), and the lizard malaria parasite studied here, *P. mexicanum* (Vardo and Schall 2007). Several outcomes are possible when multiple clones co-occur within infections. (1) The rate of increase and maximal parasitemia for both meronts and gametocytes could be the same for mixed and single-clone infections because resources limit overall parasite density. (2) Mixed-clone infections could be more productive than single-clone infections, either because each clone simply reaches its optimal density (and clones sum), or because each clone increases its rate of replication to maximize its chance of being transmitted, or all clones experience a weaker immune attack. (3) All clones suffer severe competition when they coexist in a host, and thus mixed-clone infections would be less productive, with lower rates of increase and maximal parasitemia. Superimposed on these possible events would be shifts in the first production of gametocytes as a possible mechanism for clones to compete for transmission into the vector. We sought to determine which of these possible outcomes obtain when the clonal diversity of *P. mexicanum* infections was experimentally altered.
There was no significant effect on any infection trait of *P. mexicanum* across the five main treatments (number of clones). Combining all mixed-clone infections, no difference was observed from single-clone infections for mean increase or maximal parasitemia of asexually replicating meronts, but gametocytes increased more rapidly and reached greater parasitemia in the mixed-clone infections. This means that meront replication rate must actually increase in the mixed-clone infections to convert a greater proportion to gametocytes, and that the effect is similar for two to many clones with no cumulative effect of adding additional clones. The timing of the first presentation of gametocytes in the blood did not differ by clonal diversity. An even more striking result is the more variable rate of increase and maximal parasitemia of both meronts and gametocytes in the mixed-clone infections. All of the single-clone infections fell within one standard deviation for these traits for all infections, but 39% of mixed clone infections were extreme (> 1 SD from overall mean) for at least one infection trait.

The results can now be used to evaluate possible differences in the course of infection for mixed-clone vs. single-clone infections. Final parasitemia of both gametocytes and meronts is often higher in mixed-clone infections, indicating that interference among clones does not hold final parasite density to the same or lower levels than seen in single-clone infections. Also, the higher parasitemia in mixed-clone infections is often greater than expected if total numbers are simply a sum over all clones. For example, maximal gametocyte parasitemia for two clone infections is more than twice what would be expected for a simple sum of two clones that each produced the maximal number of gametocytes (Table 7.3). The results suggest that clones of *P. mexicanum* often increase
their rate of replication and final abundance when they coexist with other genotypes of parasite. This could be a product of either some competitive race to favor each clone's transmission success or the benefit of a reduced immune attack for complex infections.

Evidence for competition among *Plasmodium* clones emerges from studies of human, rodent, and now reptile malaria parasites, although the outcome of this interaction varies enormously. For people suffering chronic infection of *P. falciparum*, sudden onset of serious illness follows arrival of a novel genotype in the infection, with no indication that specific genotypes are more virulent (Ferriera *et al.* 2002; Bruce *et al.* 2007). High clonal diversity is apparently not the driver behind these events, but simply the arrival of a new clone. Indeed, genetically diverse infections appear more successful in restricting entry of novel clones (Smith *et al.* 1999). Competition among clones of the rodent malaria parasite, *P. chabaudi*, is intense in a laboratory mouse host, but the outcome depends on specific genotypes present and their order of entry into the host (e.g. deRoode *et al.* 2003, 2005). For example, parasite replication rates and gametocyte parasitemia may be greater in mixed-clone infections, or not, depending on which parasite strains are present (Taylor *et al.* 1998; de Roode *et al.* 2003). *P. mexicanum* clones also appear to compete because a manipulative experiment shows novel genotypes are excluded from entry into an existing infection (Vardo *et al.* 2007). The experiment presented here shows that once multiple clones enter an infection and become established, some interaction often leads to changes in the rate of replication and final parasitemia of the infections.
A tidy conclusion that high clonal diversity within *P. mexicanum* infections leads to an increase in the parasite replication and parasitemia is confounded by the variation observed in the mixed-clonal infections. Only a portion of such infections were highly productive, whereas the other infections followed a course typical of single-clone infections. If either competition for transmission success or cooperation to elude the immune system results in higher replication rates and parasitemia (the remaining possible cases of those presented above), why don't all the mixed-clone infections respond? Arrival of a novel clone in some infections (with results similar to that seen for *P. falciparum* infections in humans [above]), is highly unlikely because the experimental lizards were housed in vector-proof cages, so no new clones could later have been transmitted to the lizards. Also, no new genotypes appeared in the infection based on samples taken at 30 and 80 days post inoculation. Instead, a host effect is possible, if the lizards we collected from a wild population varied in genotype or immune competency. Experiments comparing infections of *P. chabaudi* in different mouse strains, or immune system competence, reveal variation in the course of infection among treatment groups (deRoode et al. 2004; Mackinnon and Read 2003).

One trait was monotonous across all the experimental treatments, the timing of first production of gametocytes. Studies on free-living species demonstrate that increase in mortality of younger age classes leads to a concomitant shift toward early reproduction (Roff 1992). Remarkably, *P. chabaudi* follows this pattern by a shift to early production of gametocytes when infections are stressed with antimalarial drugs (Buckling *et al.* 1997). Clonal diversity itself does not appear to present a similar stress for *P.*
mexicanum. Instead, mixed-clone infections of P. mexicanum produce gametocytes at a more rapid rate and achieve higher gametocyte densities.

All of the conclusions drawn from the experiment with P. mexicanum in fence lizards would be confounded if parasite genotypes vary in their reproductive rates and maximal parasitemia. Mixed-clone infections could simply be more likely, by chance, to include one or more of these highly productive genotypes. A shortcoming of the experimental design was the impossibility of studying every individual clone alone in replicate infections. Only two clones were compared when alone and mixed, and the two-clone infections produced some that were High, but all of the replicates of the single-clone infections were Low. We used several other tactics to detect any genotype-specific effect, and found no hint that there are specific productive clones. Other studies result in conflicting views on the existence of variation in the infection characteristics of Plasmodium genotypes, with some finding such variation (Paul et al. 2004; de Roode et al. 2005) and others concluding that only the interaction of clones produces variation among infections (Babiker et al. 1998; Roper et al. 1998; Ferreira et al. 2002). Therefore, the question of variation in the infection dynamics among P. mexicanum strains remains an open, and intriguing, question.

Read and Taylor (2001) noted the scarcity of empirical tests of the common view that genetic diversity within microparasite infections should alter the dynamics of infection including rate of replication, parasite density, transmission strategy, and virulence. Cross-sectional and rare longitudinal studies of natural human malaria infections, experiments using a laboratory model of rodent Plasmodium in mice, and now the first
Manipulative study of clonal diversity for a natural parasite-host association all converge on the outcome that the number of genotype of *Plasmodium* clones within the vertebrate host does indeed play an important role in the course of those infections. However, the picture across *Plasmodium* species indicates the story is far more diverse, and more complex, than previously suspected. But, in the case of *P. mexicanum*, the finding that an intracellular protist parasite may respond to the presence of non-kin in an infection is a striking result that now begs the question of proximate mechanisms.

**Acknowledgements**

We thank the staff of the Hopland Research and Extension Center for offering their usual warm welcome and logistical support, especially C. Vaughn, R. Keiffer, and R. Timm. B. Blumberg assisted in catching and caring for lizards. The research was funding by a grants from the USA National Science Foundation and the Vermont Genetics Network to JJS.


Zoology 78:1230-1237.


insights from the epidemiology of multiple infections. Transactions of the Royal Society of Tropical Medicine and Hygiene 93:59-64.


Table 7.1. Donor infections used to initiate replicate recipient infections of the malaria parasite *Plasmodium mexicanum* in the natural lizard host, *Sceloporus occidentalis*.

Three microsatellite loci were genotyped to score the number of clones present. Alleles for each locus are labeled from smallest (shortest repeat) to longest (A to D for Locus Pmx306, 1 to 6 for Locus Pmx732, and a to c for Locus Pmx747; loci described in Schall and Vardo [2007]). Number of clones present (N clones) is reported as the largest number of alleles seen for any locus (minimum number of clones present), to the product of the number of alleles seen (maximum number of clones).

<table>
<thead>
<tr>
<th>Donor</th>
<th>Pmx306</th>
<th>Pmx732</th>
<th>Pmx747</th>
<th>N clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>A</td>
<td>5</td>
<td>c</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>E</td>
<td>5</td>
<td>a</td>
<td>1</td>
</tr>
<tr>
<td>III</td>
<td>C, F</td>
<td>2, 4</td>
<td>c</td>
<td>2-4</td>
</tr>
<tr>
<td>IV</td>
<td>B, D</td>
<td>6, 1</td>
<td>b, c</td>
<td>2-8</td>
</tr>
<tr>
<td>V</td>
<td>C</td>
<td>2, 1</td>
<td>c</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 7.2. Number of clones and donor infections used to induce replicate infections of *Plasmodium mexicanum* in the lizard host. Nine treatment groups are listed, titled based on number of clones present (three single-clone treatments, with different genotype of parasite present, and two 4-32 clone treatments with different assemblages of clones are indicated by letter). Donor infection (Table 7.1) is given, and the number of clones present presented as a range (minimum –maximum number of clones present). Treatment group 1c received blood from an infection containing 2-4 clones, but only a single clone became established. Sample size (N) of replicate infections (lizards) for each treatment is listed.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Donor(s)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>I</td>
<td>7</td>
</tr>
<tr>
<td>1b</td>
<td>II</td>
<td>7</td>
</tr>
<tr>
<td>1c</td>
<td>III</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>I+II</td>
<td>8</td>
</tr>
<tr>
<td>2-4</td>
<td>III</td>
<td>5</td>
</tr>
<tr>
<td>2-8</td>
<td>IV</td>
<td>7</td>
</tr>
<tr>
<td>4-32a</td>
<td>III-IV</td>
<td>8</td>
</tr>
<tr>
<td>4-32b</td>
<td>III+IV+V</td>
<td>14</td>
</tr>
<tr>
<td>6-14</td>
<td>I+II+III+IV</td>
<td>10</td>
</tr>
</tbody>
</table>
Table 7.3. Traits for *Plasmodium mexicanum* infections with different number of parasite genotypes (clones).

Treatment groups are labeled based on number of clones present, scored by three microsatellite loci. Results are also given for a summary of all infections containing a single clone and > 1 clone. Given here are log$_{10}$ back transformed means and (Standard Deviations), and ranges. Samples sizes for each treatment are also given (N).

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Maximum Meront Parasitemia</th>
<th>Maximum Gametocyte Parasitemia</th>
<th>Meront Rate of Increase</th>
<th>Gametocyte Rate of Increase</th>
<th>Total Meronts Produced Over Time</th>
<th>Total Gametocytes Produced Over Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a (N = 7)</td>
<td>31.4 (20.1)</td>
<td>21.0 (11.3)</td>
<td>0.92 (0.59)</td>
<td>0.69 (0.47)</td>
<td>83 (51.4)</td>
<td>66.9 (52.2)</td>
</tr>
<tr>
<td>(N = 7)</td>
<td>10-68</td>
<td>8-39</td>
<td>20-1. 90</td>
<td>0.15-1.50</td>
<td>30-179</td>
<td>21-175</td>
</tr>
<tr>
<td>1b (N = 7)</td>
<td>26.0 (20.7)</td>
<td>7.6 (6.5)</td>
<td>0.68 (0.58)</td>
<td>0.40 (0.29)</td>
<td>62.7 (51.6)</td>
<td>20.6 (22.2)</td>
</tr>
<tr>
<td>(N = 7)</td>
<td>8-66</td>
<td>1-18</td>
<td>13-1.60</td>
<td>0.03-0.75</td>
<td>27-174</td>
<td>2-57</td>
</tr>
<tr>
<td>1c (N = 3)</td>
<td>15.3 (3.2)</td>
<td>13 (10.1)</td>
<td>0.58 (0.11)</td>
<td>0.66 (0.47)</td>
<td>38.3 (10.7)</td>
<td>33.3 (21.2)</td>
</tr>
<tr>
<td>(N = 3)</td>
<td>13-19</td>
<td>4-24</td>
<td>0.45-0.65</td>
<td>0.37-1.20</td>
<td>29-50</td>
<td>11-69</td>
</tr>
<tr>
<td>2 (N = 8)</td>
<td>102.1 (139.8)</td>
<td>65.1 (95.2)</td>
<td>2.4 (2.25)</td>
<td>2.37 (2.65)</td>
<td>157.0 (257.3)</td>
<td>103.6 (113.1)</td>
</tr>
<tr>
<td>(N = 8)</td>
<td>5-350</td>
<td>4-278</td>
<td>0.28-5.74</td>
<td>0.20-6.95</td>
<td>13-737</td>
<td>12-320</td>
</tr>
<tr>
<td></td>
<td>2-4 (N = 5)</td>
<td>2-8 (N = 7)</td>
<td>4-32a (N = 8)</td>
<td>4-32b (N = 14)</td>
<td>6-14 (N = 10)</td>
<td>1 (N = 17)</td>
</tr>
<tr>
<td>----</td>
<td>------------</td>
<td>------------</td>
<td>--------------</td>
<td>----------------</td>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td></td>
<td>64.2 (70.1)</td>
<td>92.0 (134.4)</td>
<td>88.1 (73.3)</td>
<td>45.4 (48.9)</td>
<td>27.9 (32.9)</td>
<td>26.2 (18.4)</td>
</tr>
<tr>
<td></td>
<td>23.2 (13.5)</td>
<td>35.3 (32.3)</td>
<td>34.0 (27.7)</td>
<td>22.2 (19.7)</td>
<td>46.3 (63.1)</td>
<td>14.1 (10.8)</td>
</tr>
<tr>
<td></td>
<td>1.09 (1.10)</td>
<td>1.80 (2.39)</td>
<td>1.54 (1.13)</td>
<td>1.11 (1.02)</td>
<td>0.75 (0.86)</td>
<td>0.76 (0.53)</td>
</tr>
<tr>
<td></td>
<td>0.79 (0.37)</td>
<td>1.67 (1.31)</td>
<td>1.05 (0.92)</td>
<td>0.99 (0.92)</td>
<td>2.12 (3.17)</td>
<td>0.56 (0.41)</td>
</tr>
<tr>
<td></td>
<td>0.45-1.40</td>
<td>0.35-3.80</td>
<td>0.21-3.03</td>
<td>0.17-3.80</td>
<td>0.23-10.40</td>
<td>0.03-1.50</td>
</tr>
<tr>
<td></td>
<td>170.8 (148.8)</td>
<td>120.7 (201.2)</td>
<td>171.1 (91.3)</td>
<td>105.9 (110.9)</td>
<td>95.0 (128.8)</td>
<td>66.8 (47.7)</td>
</tr>
<tr>
<td></td>
<td>67.8 (39.3)</td>
<td>80.8 (76.8)</td>
<td>70.7 (45.7)</td>
<td>98.6 (147.1)</td>
<td>132.6 (176.0)</td>
<td>41.9 (42.6)</td>
</tr>
<tr>
<td></td>
<td>27-117</td>
<td>7-189</td>
<td>28-152</td>
<td>12-578</td>
<td>19-574</td>
<td>2-175</td>
</tr>
</tbody>
</table>
Table 7.4. ANOVA results for comparisons of main treatments (number of clones per infection) and nested replicate treatments within the main treatment groups for several infection traits of *Plasmodium mexicanum* in its natural vertebrate host.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Main Treatment Effects</th>
<th>Replicate Group Effect</th>
<th>1 vs. &gt;1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of increase of meront stages</td>
<td>F 8,60 = 1.24, P= 0.10</td>
<td>F 8,60 = 1.24, P= 0.74</td>
<td>F 1,67 = 1.43, P= 0.24</td>
</tr>
<tr>
<td>Max. meront parasitemia</td>
<td>F 8,60 = 1.18, P= 0.12</td>
<td>F 8,60 = 1.18, P= 0.51</td>
<td>F 1,67 = 1.56, P= 0.22</td>
</tr>
<tr>
<td>Sum of meronts over all samples</td>
<td>F 8,57 = 1.09, P= 0.33</td>
<td>F 8,57 = 1.09, P= 0.24</td>
<td>F 1,64 = 0.88, P= 0.35</td>
</tr>
<tr>
<td>Rate of increase of gametocytes</td>
<td>F 8,60 = 1.59, P= 0.09</td>
<td>F 8,60 = 1.59, P= 0.67</td>
<td>F 1,67 = 8.58, P= 0.005</td>
</tr>
<tr>
<td>Max. gametocyte parasitemia</td>
<td>F 8,60 = 1.91, P= 0.11</td>
<td>F 8,60 = 1.91, P= 0.17</td>
<td>F 1,67 = 7.74, P= 0.007</td>
</tr>
<tr>
<td>Sum of gametocytes over all samples</td>
<td>F 8,57 = 2.06, P= 0.10</td>
<td>F 8,57 = 2.06, P= 0.12</td>
<td>F 1,64 = 7.83, P= 0.007</td>
</tr>
</tbody>
</table>
**Figure Captions**

**Figure 7.1.** Four infection traits of the malaria parasite, *Plasmodium mexicanum*, within the vertebrate host (fence lizards) when experimental infections contain 1 or multiple genetic clones of haploid parasite cells. Number of clones in experimental infections is indicated by the treatment title, from 1 to 6-60, and replicate treatment groups by letters (Table 2). (A) Maximal density (parasitemia) of asexually replicating *P. mexicanum* cells (meront) found in an infection over an 80 day period given as parasites per 1000 erythrocytes. (B) Rate of increase of meront within infections in parasites per day per 1000 erythrocytes. (C) Maximal parasitemia of transmission stages (gametocytes) in infections in cells per 1000 erythrocytes (D) Rate of increase of gametocytes in cells per day per 1000 erythrocytes.
Figure 7.1
Chapter 8

Clonal diversity within infections and the virulence of a malaria parasite, *Plasmodium mexicanum*.

A. M. Vardo-Zalik and J. J. Schall

Abstract

Both verbal and mathematical models of parasite virulence predict that the genetic diversity of microparasite infections will influence the level of costs suffered by the host. We tested this idea by manipulating the number of coexisting clones of *Plasmodium mexicanum* in its natural vertebrate host, the fence lizard *Sceloporus occidentalis*. We established replicate infections of *P. mexicanum* harbouring 1, 2, 3, or >3 clones (scored using three microsatellite loci) to observe the influence of clone number on several measures of parasite virulence. Clonal diversity did not affect mortality, body growth, or production of immature erythrocytes. Blood hemoglobin concentration was highest for the most genetically complex infections (equal to that of noninfected lizards), and blood glucose levels and rate of blood clotting was highest for the most diverse infections (with greater glucose and more rapid clotting than for noninfected animals). Specific clones were not associated with virulence. In this first experiment that manipulated the clonal diversity of a natural *Plasmodium*-host system, the cost of infection with 1 or 2 clones of *P. mexicanum* was similar to that previously reported for infected lizards, but the most complex infections had either no cost or could be beneficial for the host.
Introduction

Malaria parasites (*Plasmodium* spp.) exploit two hosts during their life cycle, an insect vector (mosquito or sandfly) and a vertebrate (squamate reptile, bird, or mammal) (Valkiunas *et al.* 2005). Within the vertebrate host, the parasite meront cells undergo repeated rounds of asexual replication, and eventually some progeny develop into the transmission stages, the gametocytes. Each infection could harbour a single clone of genetically uniform meronts and gametocytes, or two to many genotypes of the parasite. Many studies, scoring a variety of genetic markers, find that infections of the human malaria parasites *P. falciparum*, *P. malariae*, and *P. vivax* vary in their number of coexisting clones. Multiclonal infections are common for these parasites, especially in locations with high transmission pressure (Gupta *et al.* 1994; Felger *et al.* 1999; Tanner *et al.* 1999; Anderson *et al.* 2000; Leclerc *et al.* 2002; Cui *et al.* 2003; Imwong *et al.* 2006; Bruce *et al.* 2007). Rare data on the *Plasmodium* of nonhuman hosts reveal a similar pattern (Vardo and Schall 2007). Ecological and evolutionary theory predicts that clonal diversity (genetic diversity) of microparasites such as *Plasmodium* will shape the virulence of the infection (Ewald 1994; Bull 1994; Nowak and May 1994; May and Nowak 1995; vanBaalen and Sabelis 1995; Frank 1996; Read and Taylor 2001; Schall 2002; Ewald 2004). This line of reasoning assumes that parasites within single-clone infections face the relatively simple challenge of reproducing at some optimal rate to ensure maximal transmission of the gametocytes while replacing cells that have been removed by the immune system. A more complex situation exists for genetically complex infections because the parasite clones must compete for host resources and the
opportunity for transmission (de Roode et al. 2005a). The outcome for multiclonal infections is predicted to be a more rapid rate of parasite replication and higher final parasite density, leading to increased costs for the host (Schall 2002). The host immune response to infection must also have its costs, which could well be more pronounced when fighting a diversity of parasite genotypes (Sheldon and Verhulst 1996, Taylor et al. 1998).

Evidence that clonal diversity is related to increased pathology for human malaria parasites is equivocal; indeed, complex infections are often associated with an asymptomatic history, with severe disease resulting only when a new genotype enters an infection (Smith et al. 1999). However, studies on human malaria parasites must by necessity be cross-sectional among infections, rather than controlled experiments. Manipulative experiments using a laboratory rodent malaria model (\textit{P. chabaudi} in inbred mice) find that genetic diversity leads to competition among clones, changes in the dynamics of infection, and increased virulence (Taylor et al. 1998; Read and Taylor 2001; Mackinnon et al. 2002; de Roode et al. 2003, 2004; Mackinnon and Read 2004; de Roode et al. 2005a, 2005b). Although these results offer valuable insight into the relationship between clonal diversity and virulence, and allow evaluation of the verbal and mathematical theory on parasite virulence, the data obtained from studies on human \textit{Plasmodium} infections lack any experimental manipulation of infections, and the rodent model lacks the coevolutionary history of a natural parasite-host association.

We examined the effects of clonal diversity on the virulence of the lizard malaria parasite, \textit{P. mexicanum}, in its natural vertebrate host, \textit{Sceloporus occidentalis}. This
parasite-host system has been studied for three decades at a site in northern California, USA (Schall 1996; Schall 2002). *P. mexicanum* is virulent for infected lizards; infection is associated with a broad array of hematological, physiological, behavioral, and reproductive deficits (Schall et al. 1982; Schall 1990a, 1990b, 1996, 2002). Genetic diversity both within and among *P. mexicanum* infections is great, with 50-88% of infections (depending on site and year) containing more than a single parasite genotype (Vardo and Schall 2007). Experimental infections can be initiated successfully by blood transfer that controls the number of parasite clones (Osgood et al. 2003; Vardo et al. 2007). Thus, *P. mexicanum* in its lizard host provides an excellent opportunity to test predictions of the theory on parasite virulence, and also to compare the costs of infection itself with the added influence of clonal diversity.

We established replicate experimental infections containing one to many parasite clones (scored using recently discovered variable microsatellite markers for *P. mexicanum* (Schall and Vardo 2007), and asked if clonal diversity was associated with several measures of virulence relevant for the lizard host: mortality, changes in mass, blood hemoglobin and blood glucose concentrations, production of immature red blood cells, and tendency for the blood to clot. We also asked if virulence was associated with specific parasite genotypes.

**Methods**

The experiment was conducted at the University of California Hopland Research and Extension Center, near the town of Hopland, in southern Mendocino County, California, USA. Naturally infected lizards were captured from five locations on the 2169 ha field
station (all within 1.2 km of 39° 00' 02" N, 123° 04' 39" W) to serve as blood donors to initiate experimental infections. Genotypes for each infected lizard were analyzed at three microsatellite loci (Pmx 306, 747 and 732) using PCR primers and conditions presented in Schall and Vardo (2007), and the labeled PCR product run on an ABI Prism instrument and results analyzed using GeneMapper software (ABI). Parasite prevalence was very low during the study, with only ~ 6% of lizards infected. Single-clone infections with high parasitemia of asexually replicating meront stages (at least 25 meronts/1000 RBC) were rare in the early warm season. Therefore, only five suitable donors were identified from 484 lizards sampled (Table 8.1). Single-clone donor infections were those that were scored as one allele for each locus (two of the donor infections). One donor infection was scored as two clones based on two alleles at one locus and one each at the other two. Two more donor infections had two alleles at more than one locus. Therefore, we estimated the clonal diversity within an infection as the maximum number of alleles observed at any of the three microsatellite loci. This estimate represents the minimum number of clones present within an infection and is proposed to be an unbiased estimate of clonal diversity (Anderson et al. 2000; Ferreira et al. 2002; Bogreau et al. 2006; Bruce et al. 2007; Ferreira et al. 2007; Vardo and Schall 2007).

Seventy-eight noninfected adult male lizards with a snout to vent length (svl) > 55 mm were collected from locations at the field station (N= 2; 39° 02' 05" N, 123° 05' 49" W and 38° 59' 27" N, 123° 05' 27" W) where P. mexicanum infection in the lizards has been absent for many years. Examination of blood smears, and a sensitive PCR-based protocol that detects infections subpatent in blood smears confirmed the recipient lizards
were not already infected with *P. mexicanum* (Perkins et al. 1998; Vardo et al. 2005). Using these standards, a control group of 21 uninfected lizards was maintained throughout the study (below) and none of these lizards converted to infection.

Experimental infections were initiated with blood from one to four donors using the protocol of Vardo-Zalik and Schall (2008). Briefly, blood was taken from each donor lizard, and the number of parasites per ml of blood calculated based on counts of parasites per 1000 erythrocytes examined in stained blood smears and erythrocytes/ml of blood estimated using a Hausser counting chamber. Blood from donor lizards was then mixed with phosphate buffer saline (PBS), and the blood/PBS mixture was diluted to contain approximately $200 \times 10^3$ total meronts. For multiclonal infections initiated with blood from $>1$ donor, each donor supplied similar numbers of asexual parasites, with the total number of parasites injected held constant ($200 \times 10^3$). Infections were started via intraperitoneal injections with 20 ml of the donor blood/PBS mixture.

Using the minimum number of clones rule described above, four treatment groups received blood containing 1, 2, 3, or $>3$ clones (4 - 6 clones). This range in clonal diversity is similar to that seen within natural infections of *P. mexicanum* (Vardo and Schall 2007). Each experimental infection was genotyped on day 30 when infections become patent in the blood and day 80 at the end of the experiment. Not all introduced clones became established (as observed previously in such experiments with *P. mexicanum*; Vardo et al. 2007), so the final sample sizes within each treatment group were determined by the genotyping results from the recipient infections. Three replicates of Treatment Group 1 (single-clone recipient infections) received blood from donors I (N
= 8), II (N = 8), or III (N = 3) (Table 8.1). Donors I and II harbored single-clone infections, and donor III a two-clone infection, but only one of those was detected in the recipients. Four replicates of Treatment Group 2 received blood from donors I+II (N = 8), III (N = 5), IV (N = 8) and III+IV+V (N = 5). Two replicates of Treatment Group 3 received blood from donors III+IV (N = 1) and donors III+IV+V (N = 4). Again, assignment of lizards to Treatment Groups 2 or 3 was based on the number of clones that became established. Three replicates of Treatment Group >3 received blood from donors III+IV (N = 7), donors I+II+III+IV (N = 10), and donors III+IV+V (N = 11). Thus, a total of 78 infections were initiated into treatments with 1 (N = 19 total), 2 (N = 26), 3 (N = 5), and >3 (N = 28) clones. The control group of 21 lizards, judged not infected by the criteria given above, was used to monitor changes in virulence measures due to captivity.

For the three-month experimental period, lizards were housed outdoors in 12 vector-proof cages, with lizards housed by replicate to insure that parasite clones could not be passed by means other than vector bites among the treatments (Schall and Smith 2006). The cages were hung from overhead wire in a 5 x 2 m area, and each week were randomly moved within that array. Drops of blood were drawn from toe clips to make a blood smear and a dried/frozen blood drop every 10 days. On those dates, lizard mass was determined. Lizards were fed mealworms or crickets to satiation and were showered with cool water hourly from 13:00-19:00 when temperatures exceeded 29°C.

Virulence measures recorded were chosen based on previous studies on the effect of *P. mexicanum* on fence lizards (reviews in Schall 1996, 2002). These measures were: (1)
Mortality. Lizards were examined several times each day to detect any that had died. (2) Change in body mass. Lizards were weighed every ten days when blood smears were taken. (3) Proportion of immature erythrocytes. The blood smear made on day 80 post injection was stained with Giemsa and 100 erythrocytes examined from all areas of the smear under 1000x. Immature cells are distinguished by a larger, rounder nucleus and their darker, more blue color. This measure was taken three times for each lizard, and the mean of all three values was used. (4) Blood hemoglobin. On day 80 post injection, blood hemoglobin level was determined by the cyanmethemoglobin method in which the concentration of hemoglobin is linearly related to absorbance at 540 nm detected using a spectrophotometer (Schall 1990a). (5) Blood glucose. Glucose was measured using a FreeStyle glucose monitor which utilizes a glucose dehydrogenase pathway to estimate glucose concentrations in whole blood (Voituron et al. 2002). (6) Blood clotting. In humans, Plasmodium infection is associated with changes in blood clotting (Kaul et al. 1991; Gosh and Shetty 2008), and in preliminary studies we also noted differences in clotting rate for infected and noninfected lizards. Therefore, we scored each lizard on day 80 post injection as "rapidly clotting blood" if the blood at once formed a blood clot in a heparinized capillary tube (used to take blood for the hemoglobin measures). All procedures followed a protocol approved by the University of Vermont Institutional Animal Care and Use Committee.

Data were analyzed using the statistical programs JMP 6.0 and Statview 5.01. All analyses were performed as nested ANOVA (replicates nested within clonal groups), $\chi^2$, or t-tests with a significance level set at $P < 0.05$. 

170
Results

All 78 recipient lizards established infections within 1 month, except one lizard that did not show parasites in the blood until day 70 post injection. Experiment-wide mortality was 12/78 (15%) infected lizards and 2/21 (10%) noninfected controls, with no significant difference for infected vs. not infected, nor among treatment groups for infected lizards ($\chi^2$ tests, $P > 0.05$). This result is highly unlikely to be a Type II error due to small sample size because sample size of infected/noninfected would need to each be 400 for a significant result (for 15% vs. 10% mortality). Mortality reduced the total sample size for other analyses to 66 experimentally infected lizards (17 1-clone, 19 2-clone, 5 3-clone and 25 >3-clone) and 19 controls.

Summary data for other virulence measures are given in Table 8.2. Almost all lizards gained mass during the experiment. Mass change did not differ for infected vs. control lizards (t-test, $P = 0.88$), nor among treatment groups for the infected lizards (ANOVA, $P = 0.98$), with no effect of replicates within treatment groups ($P = 0.44$). Proportion of immature erythrocytes also did not differ for treatment groups (ANOVA, $P = 0.18$), with no replicate effect ($P = 0.71$), but was significantly higher in infected individuals when compared to the noninfected controls (t-test, $P = 0.0001$). Blood clotting was significantly more common in lizards within the >3-clone treatment group vs. any other treatment, including the control lizards. To allow a $\chi^2$-test, data for treatment groups 1, 2, and 3 were combined to yield expected values $> 5$ ($\chi^2 = 13.41$, $P = 0.009$). A Posthoc Cell Contributions test indicated it was only the > 3 clone treatment revealed the higher number of rapidly clotting samples. Severe blood clotting prevented determination of
blood hemoglobin concentration in 1 control and 18 experimental lizards. Hemoglobin concentration differed among treatment groups, with treatment group 2 having lower mean hemoglobin concentration than the >3 treatment (ANOVA $P=0.002$; Tukey’s HSD). A replicate effect within treatments was also observed ($P=0.03$) within the 2-clone replicate groups. Control lizards had similar levels of hemoglobin in their blood as the 3 and >3 clone treatment groups, significantly higher than the 1 and 2-clone treatments (ANOVA $P=0.0001$; Tukey’s HSD). Glucose concentration also differed among treatment groups, with the infections with fewer clones (treatments 1 and 2) having lower glucose levels than the 3 and >3 treatments (ANOVA $P=0.005$; Tukey's HSD), with no replicate effect ($P=0.16$). Blood glucose levels were similar for the control group and infection treatments 1 and 2, but lower than for treatments 3 and >3 (ANOVA, $P=0.0001$, Tukey’s HSD).

Parasite genotypes were inspected to determine if specific alleles and/or multilocus genotypes were associated with the differences seen between the 1 and 2 compared to the 3 and >3 clone infections. Four of the five donor lizards supplied blood for multiple treatment groups, including the single and >3 clone groups, so some of the multilocus genotypes were found in all treatment groups. We then scored the predominant allele at each locus for each infection (the allele that produced the highest peak on the pherogram produced by the genetic analyzer). For locus Pmx306, four alleles were scored as predominant among infections and three alleles were predominant ones for locus Pmx732. All of these alleles were predominant in infections across all treatment groups. For locus Pmx747, three alleles were predominant among infections, but one was unique
to treatment group 1 with a single clone, occurring in 40% of those infections. Treatment
groups 1 and 2 did not differ for any virulence measure, so that allele is unlikely to be a
driver of differences between the low and high clonal diversity treatments.

**Discussion**

Prior to this study on *Plasmodium mexicanum*, two lines of evidence cast light on the
role of clonal diversity on the virulence of malaria parasites: the observational field
studies on natural *P. falciparum* infections (with no manipulation of clonal numbers) and
experiments on the rodent malaria parasite *P. chabaudi* in a laboratory model system.
Results from the studies on *P. falciparum* conclude that high clonal diversity may or may
not lead to severe symptoms, and that host factors are just as important as parasite effects
in determining pathology (Gupta *et al.* 1994; Felger *et al.* 1999; Tanner *et al.* 1999;
Ofosu-Okyere *et al.* 2001; Leclerc *et al.* 2002; Cui *et al.* 2003). Most striking in these
studies is that complex *P. falciparum* infections may be asymptomatic, and severe illness
results only when novel clones enter an established infection (Felger *et al.* 1999; Smith *et
al.* 1999). The laboratory rodent malaria system allows experimental manipulation of
clonal diversity, as well as characterization of specific parasite genotypes. The results
demonstrate that the virulence of *P. chabaudi* differs by both parasite genotype and
clonal diversity within infections. In the experiments, mice containing multiclonal
infections of *P. chabaudi*, usually harboring only two clones, often have increased host
anemia, reduced body mass, and other pathologies (Taylor *et al.* 1998; Mackinnon and
Read 1999; Read and Taylor 2001; Mackinnon *et al.* 2002; de Roode *et al.* 2003;
Mackinnon and Read 2003; de Roode *et al.* 2004; Mackinnon and Read 2004; de Roode
et al. 2005a, 2005b). The higher virulence of genetically complex infections may be based, at least in part, on the higher cost of mounting an immune attack against multiple parasite clones (Taylor et al. 1998). A limitation of the rodent system is the artificial combination of a parasite of African thicket rats in a laboratory mouse host, a parasite-host association that has not coevolved.

We present here the first manipulative study of the effects of clonal diversity on several virulence measures for the vertebrate host of a natural *Plasmodium* association. The results present a complex, and unexpected, relationship between the clonal diversity of infections and the effects on the lizard host. Manipulating the number of parasite clones within treatments, revealed no effect of infection itself or clonal diversity of infection on change in body mass or mortality when compared to noninfected lizards over an 80 day period. The proportion of immature erythrocytes, a measure of the rate of replacement of red blood cells, was higher for infected than noninfected lizards, but with no additional effect of clone number. The surprising finding was that hemoglobin concentration was highest for the noninfected lizards and those infected with >3 clones, and infections with 3 or >3 clones were associated with the highest glucose levels in the lizard’s blood. Thus, for increase in body mass, mortality rates, turn-over of blood erythrocytes, hemoglobin concentration, and glucose levels, the highest clonal diversity either had no additional negative effects or actually appeared to have a positive effect on the lizards. And last, very rapid clotting of the blood was associated with infections with >3 clones. Changes in blood clotting resulting from *Plasmodium* infection are well known for human hosts, but evaluating these as positive or negative for the health of the
host has proven difficult (Newton et al. 2004; Ghosh and Shetty 2008). The overall conclusion is that infection with 1 - 2 *P. mexicanum* clones may have negative consequences for fence lizards, but highly complex infections induce little harm or may actually benefit the lizard (provided high glucose levels and rapid clotting are beneficial).

We can now compare these results with prior studies on *P. mexicanum* in natural infections followed in free-ranging fence lizards. Infected lizards in their home territory are just as able to harvest prey as noninfected lizards (Eisen and Schall 1997) and have similar growth rates (svl, rather than body mass was measured) (Ressel and Schall 1989). Our results agree that infection does not reduce growth rate, and also that clonal diversity of infections does not influence growth. All lizards had ready access to plentiful insect prey in their cages, so the results show that the parasite does not interfere with assimilation of these easily captured food items. Past studies of mortality rates of infected and noninfected fence lizards present conflicting results, with some showing no increase in mortality for naturally infected free-ranging animals (Bromwich and Schall 1986; Eisen 2001) or an increase in mortality for free-ranging infected lizards or those brought into captivity (Schall 1983; Schall 2002). Here we found no increase in mortality for infected animals, and no influence of clonal diversity. However, in a transmission study performed the year after this experiment, we found that infections with 3 or more clones were associated with a significantly higher mortality rate in captivity when compared to other treatments and control lizards (unpublished data).

Naturally infected fence lizards present a higher proportion of immature erythrocytes in the peripheral circulation. Such cells contain less hemoglobin, and thus hemoglobin
concentration in the blood is lower in infected lizards (Schall et al. 1982; Schall 1990a, 1990b, 1996; Schall 2002). A similar result for production of immature erythrocytes was seen in the infected vs. noninfected lizards in this study, but with no difference seen among the treatment groups of infected lizards. Thus, destruction of damaged and/or infected erythrocytes and the subsequent increased production of new blood cells by the host is a product of infection, rather than infection complexity and may be a generalized immune response to *Plasmodium* infection. A curious result is the similar highest concentration of hemoglobin in the blood of noninfected lizards and those infected lizards carrying three or more clones, despite a higher proportion of immature erythrocytes in all infected treatments. A higher hemoglobin concentration allows more rapid delivery of oxygen to the lizard's tissues, and a greater running stamina, both likely to be ecologically important measures of health (Schall 2002). Previous findings that naturally infected lizards suffer lower hemoglobin concentrations is likely to be a consequence of very high clonality being uncommon in the population of infected lizards at the site (Vardo and Schall 2007).

Dunlap and Schall (1995) found that naturally infected fence lizards carry lower concentrations of blood glucose. This conflicts with the experimental study that found no reduction in glucose for lizards with 1 or 2 parasite clones, but an increase in glucose for infections with highest clone numbers (3 and > 3) when compared to the noninfected control lizards. This discrepancy could be attributed to differences in methodology, because our lizards were kept in captivity for 80 days, while the previous study kept lizards for a short period of time (< 24 hrs.). Increased stress from captivity could result
in a lower blood glucose level in lizards as observed in our noninfected controls. Wild caught, noninfected lizards have mean blood glucose concentration upon capture of 270mg/dl (Dunlap and Schall 1995), but in our study, this was 25% lower (206 mg/dl) for noninfected control lizards after 80 days of captivity. Wild caught infected lizards (with no information on clonal diversity of the infections) had a mean glucose level of 243 mg/dl, close to that seen in the experimental infections carrying 3 or > 3 clones, but higher than those with 1 or 2 clones (Table 8.2). This suggests that, despite the stress of captivity reducing blood glucose in these lizards, diverse infections may actively manipulate the host into releasing more glucose into the peripheral blood. *Plasmodium* parasites feed on host glucose, so any increases in blood glucose concentration could positively affect the parasite’s reproductive rate and transmission success (Mehta *et al.* 2005). Lizards naturally have higher blood glucose levels than other reptiles (Khanna and Kumar 1975; Chandavar and Naik 2004) suggesting that higher glucose concentrations in diverse infections may cause less stress on the lizard host, and thus diverse infections may be beneficial to both parasite and lizard.

An intriguing result from this experiment was the increase in rapid blood clotting among infected lizards with highest clonal number. The blood of 5% of the control noninfected lizards clotted, and 17% of infections with 1, 2, or 3 clones (with no significant difference), but 44% of infections carrying > 3 clones clotted. The mechanism involved in the clotting of infected blood is unknown. Humans infected with *P. falciparum* experience reduced numbers of platelets in the blood, but without the expected excessive bleeding response, and perhaps even a fleeting increase in clotting
during injury (Newton et al. 2004). Platelets are not present in lizard blood, but their function is performed by thrombocytes, which could be increased in either numbers or activity for genetically complex infections, a possibility of significance for understanding the physiology of reptilian blood. Also, it is unclear if the increase in clotting is driven by host effects or direct action of the parasite. Interpretation of more frequent clotting as beneficial or harmful for the lizard is problematic, because rapid blood clotting may benefit the lizard after injury, but if clots form within the circulation, diverse infections could result in major injury to organs.

Overall, our findings suggest that low diversity infections are harmful for the lizard host; such infections are the most common at the site (Vardo and Schall 2007). However, lizards with highly diverse infections are similar to noninfected animals in blood hemoglobin, and have the highest concentration of blood glucose and the highest rate of blood clotting. Thus, a high genetic diversity of parasites within an infection could be viewed as beneficial to the lizard host, a striking finding if true. The results for *P. mexicanum*, a parasite of lizards, *P. falciparum* of humans, and *P. chabaudi* in laboratory mice, all demonstrate that parasite clonal diversity is important for the consequences of infection, but the biology is far more complex than assumed in standard theoretical models of parasite virulence.
Acknowledgements

We thank the staff of the Hopland Research and Extension Center for offering their usual warm welcome and logistical support, especially C. Vaughn, R. Keiffer, and R. Timm. B. Blumberg (2005), N. Zalik (2006), S. Reece (2006) and M. Robinson (2006) assisted in catching and caring for lizards. The research was funding by a grants from the USA National Science Foundation and the Vermont Genetics Network to JJS.
Literature Cited


Table 8.1. Donor infections used to initiate replicate recipient infections of the malaria parasite *Plasmodium mexicanum* in the natural lizard host, *Sceloporus occidentalis*.

Three microsatellite loci were genotyped to score the number of clones present. Alleles for each locus are labeled from smallest (shortest repeat) to longest (A to D for Locus Pmx306, 1 to 6 for Locus Pmx732, and a to c for Locus Pmx747; loci described in Schall and Vardo [2007]). Number of clones present (N clones) is reported as the largest number of alleles seen for any locus.

<table>
<thead>
<tr>
<th>Donor</th>
<th>Pmx306</th>
<th>Pmx732</th>
<th>Pmx747</th>
<th>N clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>A</td>
<td>5</td>
<td>c</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>E</td>
<td>5</td>
<td>a</td>
<td>1</td>
</tr>
<tr>
<td>III</td>
<td>C, F</td>
<td>2, 4</td>
<td>c</td>
<td>2</td>
</tr>
<tr>
<td>IV</td>
<td>B, D</td>
<td>6, 1</td>
<td>b, c</td>
<td>2</td>
</tr>
<tr>
<td>V</td>
<td>C</td>
<td>2, 1</td>
<td>c</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 8.2. Summary data (mean ± st. dev) and range for five virulence measures for western fence lizards (*Sceloporus occidentalis*) experimentally infected with the malaria parasite, *Plasmodium mexicanum*. Treatment groups are infections containing 1, 2, 3 or > 3 genetically distinct clones and a control, noninfected group. Measures are determined on day 80 after the experimental infections were initiated. Measures are weight gain over the 80 day experiment, glucose concentration in the blood, a measure of blood hemoglobin concentration (absorbance at 540 nm), proportion of immature erythrocytes and number of animals with blood that clotted very rapidly.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight Gain (grams)</th>
<th>Glucose Concentration (mg/dl)</th>
<th>Hemoglobin (absorbance)</th>
<th>Prop. immature erythrocytes</th>
<th>Clotting</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 clone</td>
<td>2.52 ± 0.68</td>
<td>207.12 ± 41.11</td>
<td>0.11 ± 0.03</td>
<td>0.75 ± 0.11</td>
<td>5/17</td>
</tr>
<tr>
<td>N= 17</td>
<td>0.90-3.70</td>
<td>157-324</td>
<td>0.08-0.16</td>
<td>0.57-0.94</td>
<td></td>
</tr>
<tr>
<td>2 clone</td>
<td>2.21 ± 0.61</td>
<td>187.53 ± 40.02</td>
<td>0.08 ± 0.03</td>
<td>0.81 ± 0.11</td>
<td>2/19</td>
</tr>
<tr>
<td>N= 19</td>
<td>1.10- 3.30</td>
<td>127-280</td>
<td>0.03- 0.15</td>
<td>0.50-0.94</td>
<td></td>
</tr>
<tr>
<td>3 clone</td>
<td>2.30 ± 0.43</td>
<td>261.40 ± 32.63</td>
<td>0.11 ± 0.03</td>
<td>0.79 ± 0.10</td>
<td>0/5</td>
</tr>
<tr>
<td>N= 5</td>
<td>1.70-2.90</td>
<td>226-309</td>
<td>0.07-0.15</td>
<td>0.66-0.91</td>
<td></td>
</tr>
<tr>
<td>&gt; 3 clone</td>
<td>2.44 ± 0.73</td>
<td>242.60 ± 45.71</td>
<td>0.15 ± 0.04</td>
<td>0.73 ± 0.09</td>
<td>11/25</td>
</tr>
<tr>
<td>N= 25</td>
<td>1.10-4.10</td>
<td>129-303</td>
<td>0.07-0.18</td>
<td>0.57-0.94</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>2.36 ± 0.64</td>
<td>205.53 ± 29.76</td>
<td>0.15 ± 0.04</td>
<td>0.26 ± 0.12</td>
<td>1/19</td>
</tr>
<tr>
<td>N=19</td>
<td>0.90-3.5</td>
<td>154-256</td>
<td>0.10-0.24</td>
<td>0.07-0.60</td>
<td></td>
</tr>
</tbody>
</table>
Appendix A

Identification of microsatellite markers in *Plasmodium mexicanum*, a lizard malaria parasite that infects nucleated erythrocytes

Jos. J. Schall and Anne M. Vardo

Abstract

Reptile and bird hosts of malaria parasites (*Plasmodium*) have nucleated erythrocytes. Infected blood thus contains a mix of abundant host and scant parasite DNA which has prevented identification of *Plasmodium* microsatellites. We developed a protocol for isolation of microsatellite markers for *P. mexicanum*, a parasite of lizards. The ATT repeat was common in the genome of *P. mexicanum*, but most (87%) of these repeats were exceptionally long (50 - 206+ repeats). Seven microsatellite markers with PCR primers are described. The protocol should allow discovery of microsatellites of malaria parasites (with AT-rich genomes) infecting bird and reptile hosts.

Introduction

Within the vertebrate host, infections of a malaria parasite (*Plasmodium*) may consist of a single genotype of haploid cells (single clone) or several genotypes. Clonal diversity appears important for the parasite's life history and transmission biology (deRoode *et al.* 2005), and therefore assaying the number of genotypes within infections is important for studies of the ecology of *Plasmodium*. Full-genome sequencing has revealed numerous
microsatellites in *P. falciparum*, the major human malaria parasite, that are useful for studies in ecological genetics (Awadalla *et al.* 2001). However, most *Plasmodium* species are parasites of birds and reptiles. These parasites are phylogenetically distant to the *Plasmodium* of mammals (Perkins & Schall 2002), and we found that 20 primer pairs for *P. falciparum* microsatellites failed to produce product for *P. mexicanum*, a parasite of fence lizards (*Sceloporus occidentalis*). Use of standard techniques for discovery of microsatellites is unproductive for the saurian/avian malaria parasites because the vertebrate hosts have nucleated erythrocytes. In a blood sample, concentration of host DNA is much greater than that of the haploid parasite. Here we report the first identification of microsatellite markers for a malaria parasite infecting a vertebrate host with nucleated erythrocytes, *P. mexicanum*. The method (details at www.uvm.edu/~jschall/malaria.html) is based on that of Hamilton *et al.* (1999).

**Materials and Methods**

The genome of most malaria parasites appears to be highly AT-rich (*P. falciparum, P. yoelii, P. berghei, P. gallinaceum*, but not *P. vivax*). Such AT-rich DNA will denature near standard PCR extension temperature (72° C) (Su *et al.* 1996). Therefore, to favor PCR amplification of *P. mexicanum* DNA, a low denaturation temperature (84° C) and low extension temperature (60° C) were used. Genomic DNA was enriched for an ATT repeat, the most common motif for *P. falciparum* microsatellites (Su & Wellems 1996), but one that is relatively rare in the vertebrate genome (BLAST search).
DNA was extracted from blood of fence lizards heavily infected with *P. mexicanum*, and was digested with Sau3AI. Fragments of 400 to 1200 bp were isolated from an agarose gel. Damage to the low concentration of parasite DNA was prevented by cutting off the two edge ladder lanes from the gel and staining only these in ethidium bromide. The ladder lanes were replaced on the gel with a piece of aluminum foil under the target DNA lanes. The gel was examined under UV illumination and the proper section removed. DNA was purified from the gel, and then ligated onto a double-stranded linker that matched the Sau overhang. The linker was designed for a low PCR annealing temperature and was produced from: LinkA = 5’ GATATACTAATGAATGTTGG 3’; LinkB = 5’ GATCCCAACATTCATTAGTATATC 3’. For PCR amplification of the DNA, only LinkA was needed as the primer using the program: 84° C 2 min, 32 cycles of 84° C 1 min, 52° C 10 sec, 48° C 10 sec, and 60° C 4 min, followed by a final extension of 60° 4 min.

Three enrichments for the target microsatellite used a (TAA)$_{10}$ biotinylated oligonucleotide. The PCR product was hybridized with the labeled repeat, and avidin beads (Vector) captured the DNA fragments attached to the biotin-labeled repeat. After each round of enrichment, the DNA was cleaned and amplified again using the above PCR program. The final PCR product was used as template for TOPO (Invitrogen) cloning. Cloned fragments were sequenced (ABI BigDye). Selection of sequences for further study was based on a high AT-rich flanking regions and a BLAST search that found at least 15 bases overlap with *P. falciparum* (the latter method was a poor indicator because of the divergence of the two *Plasmodium* species). Primers were designed and
the locus was scored as "parasite" if PCR product produced a band for lizards infected with *P. mexicanum*, but not for uninfected lizards.

**Results and Discussion**

A total of 156 cloned fragments were sequenced, with 86% revealing the ATT repeat. Of sequences containing the repeat, 15% were identified as from the lizard genome. Fully 99 sequences containing the microsatellite revealed a very long repeat. Of 65 with sufficient flanking region for study, 53 were unique sequence, indicating a true abundance of these long repeats. Long repeats entirely contained in sequences ranged from 50 - 176 x ATT (Median = 83x; N = 39), and those that ran off at the end of the sequence ranged from 48 - 206 x ATT (Median = 93). We suspect these are from the parasite's genome. A BLAST search of the *Mus* and *Gallus* genomes, found no ATT repeats as long as 50x, whereas such repeats are present for *P. falciparum* (although the ATT 100x repeat is rare). We obtained sufficient flanking sequence to perform a successful BLAST search for 16 of the long repeats and all matched one or more species of *Plasmodium*. We were able to design primers for only one sequence with a long repeat, and confirmed this locus (Pmx 308) was parasite. All of the sequences identified as lizard contained a far shorter repeat (12 - 35x; median = 15). Subsequent to the described search, we altered the protocol in a way to avoid the expense of sequencing long repeats. A third primer, the (TAA)$_{10}$ without the biotin molecule, was added to the PCR mixture when amplifying the cloned DNA fragments. On an agarose gel, clones that did not contain a repeat produced a single, sharp band. Clones with a long repeat
produced a long smear because the third primer annealed at many places on the repeat region. Clones with a short repeat produced a short smear.

Of 14 microsatellite loci from the *P. mexicanum* genome, 7 were duplicates. Primers were designed for amplification of the remaining 7 loci, (Table A1). Six loci displayed useful variation, providing markers for study of the clonal diversity of the parasite (Table A1). The locus with only two alleles, Pmx308, was also the longest (primary allele = 74x repeat).

Despite the low concentration of parasite DNA in blood extract, the protocol was highly successful in enriching for *P. mexicanum* DNA containing the ATT microsatellite. However, the genome of *P. mexicanum* appears unusual in containing primarily very long ATT repeats. The protocol should be useful in locating microsatellites in other malaria parasites of birds and reptiles, provided those species have a highly AT-rich genome.

**Acknowledgements**

We thank Guiyun Yan and C. W. Kilpatrick for many suggestions, and Kim Kaufhold for assistance in the laboratory. The study was funded the Vermont Genetics Network and Vermont EPSCoR.
Literature Cited


Table A1 (next page). Primer sequences for microsatellite markers of *Plasmodium mexicanum*. The expected allele size is presented for a given repeat length. PCR amplification is achieved with the program 94° C for 2 min, followed by 32 cycles of 94° C for 1 min, annealing (given in table), 60 ° C for 1 min, followed by a final extension of 60° C for 4 min. For fragment analysis, the forward primer was dye-labeled. Number of alleles given is based on an initial survey of 100 infected fence lizards from a site in Mendocino County, California, near the town of Hopland. Many infections contained > 1 allele (clone of haploid parasite). All loci except Pmx308 revealed substantial diversity among infections. For these loci, half or more of alleles were at least 5% frequency among all alleles scored. An allele association analysis revealed no evidence for linkage of the loci.
<table>
<thead>
<tr>
<th>Locus</th>
<th>Forward/Reverse primer 5' - 3'</th>
<th>Annealing conditions</th>
<th>No. alleles</th>
<th>Size</th>
<th>GenBank no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pmx306</td>
<td>GATCACATTTTGCTATTTTAGTATT AACTTTTGATTCTTTCTATAACAG</td>
<td>60° C/10 sec + 50° C 20 sec</td>
<td>16</td>
<td>203 @ 27x</td>
<td>DQ503416</td>
</tr>
<tr>
<td>Pmx308</td>
<td>CTTTTGTTATAATTATTCATATTTT CAATAATAATAAATATAAATAACAACA</td>
<td>47.5° C/30 sec + 60° C/20 sec</td>
<td>2</td>
<td>312 @ 74x</td>
<td>DQ503422</td>
</tr>
<tr>
<td>Pmx328</td>
<td>TATTATTTAAGTTTTGAATGG CTTCTTTACTTTACAAAAAT</td>
<td>42.5° C/30 sec + 43.6/20 sec</td>
<td>5</td>
<td>261 @ 18x</td>
<td>DQ503417</td>
</tr>
<tr>
<td>Pmx710</td>
<td>GCCGTCTTTATGAATTAAGTGAACAAG CATTTTGCTATTTTAGTATTTTCTA</td>
<td>55° C/50 sec</td>
<td>16</td>
<td>318 @ 16x</td>
<td>DQ503418</td>
</tr>
<tr>
<td>PMx732</td>
<td>CAGGTAGATATTTTTTGATG GATGAAATGAGATAAAATCC</td>
<td>56° C/50 sec</td>
<td>17</td>
<td>255 @ 22x</td>
<td>DQ503421</td>
</tr>
<tr>
<td>Pmx747</td>
<td>CACAAATTCAAGATAATTCCAAAG TCTTTTCGAGACATATTATTGC</td>
<td>51° C/30 sec + 49° C/20 sec</td>
<td>13</td>
<td>182 @ 24x</td>
<td>DQ503420</td>
</tr>
<tr>
<td>Pmx839</td>
<td>CATTGAGAATAATCCGTTAAG GGGACCATAATGAAATTTGATTTC</td>
<td>47° C/30 sec + 44° C/20 sec</td>
<td>14</td>
<td>255 @ 57x</td>
<td>DQ503419</td>
</tr>
</tbody>
</table>
Appendix B

Lab Work Guidelines for Microsatellite Project

1. DNA extraction Protocol: Qiagen DNeasy Kit
   - Leave in 55 degree water bath for AT LEAST 2.5 hours and up to 24 hrs.
   - Use 50 µl AE for final elution step

2. Genotype PCR: using Ready to go Beads (GE Healthcare)
   - Recipe: 1µl labeled Forward Primer (10 µM)
     1µl Reverse Primer (10µm)
     5µl DNA
     18µl H2O
   - Be sure to use appropriate Program for the specific primer pair you are testing.
   - ALWAYS USE A WATER NEGATIVE CONTROL!
   - Run Samples out on gel
     - If using large well gel (10 samples/lane), use 5µl PCR product to load
     - If using small well gel (15 samples/lane), use 4µl PCR product to load

<table>
<thead>
<tr>
<th>Marker Name</th>
<th>Forward Labeled (10µm)</th>
<th>Reverse (10µm)</th>
<th>PCR Program</th>
<th>Size Range</th>
<th>Dye</th>
<th>FILTER (Cancer Facility)</th>
</tr>
</thead>
<tbody>
<tr>
<td>306</td>
<td>306 F</td>
<td>306 R</td>
<td>Locus 306</td>
<td>150-350</td>
<td>6-FAM</td>
<td>D OR G</td>
</tr>
<tr>
<td>328</td>
<td>328 F</td>
<td>328 R</td>
<td>Locus 328</td>
<td>200-500</td>
<td>NED</td>
<td>D OR G</td>
</tr>
<tr>
<td>839</td>
<td>839 F</td>
<td>839 R</td>
<td>Locus 839</td>
<td>200-400</td>
<td>NED</td>
<td>D OR G</td>
</tr>
<tr>
<td>747</td>
<td>747 F</td>
<td>747 R</td>
<td>Locus 747</td>
<td>150-300</td>
<td>HEX</td>
<td>D ONLY</td>
</tr>
<tr>
<td>732</td>
<td>732 F</td>
<td>732 R</td>
<td>Locus 732</td>
<td>150-350</td>
<td>NED</td>
<td>D OR G</td>
</tr>
<tr>
<td>739 (old 710)</td>
<td>710 F</td>
<td>739 R</td>
<td>Locus 739</td>
<td>200-400</td>
<td>PET</td>
<td>G ONLY</td>
</tr>
</tbody>
</table>

(710 PRIMER IS ALSO LABELED 710B- THEY ARE THE SAME)
Dilutions and Multiplexing for Cancer Facility UVM

For a single Locus:
Total volume in each well on a 96-well plate will vary depending upon concentration of product.
-If your product showed a band on your gel: 5ul PCR + 90ul H2O
-If your product did not show a band: 5ul PCR + 45ul H2O

Multiplexing:
You can multiplex 2 or 3 loci in a single well (PCR separately).

<table>
<thead>
<tr>
<th># Loci</th>
<th>Multiplex Filter D</th>
<th>Multiplex Filter G</th>
<th>Works best alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>306+747 or 747+839 or 306+839</td>
<td>328+739</td>
<td>732</td>
</tr>
<tr>
<td>3</td>
<td>306+747+839</td>
<td>328+739</td>
<td>732</td>
</tr>
</tbody>
</table>

Dilutions For 2 Loci:
*see the above chart for multiplexing primer pairs and filter info.

Total volume in each well of the 96 well plate should be 50µl.
If both samples produced no band on the gel:
5 µl PCR sample 1 (locus 747)
5 µl PCR sample 2 (locus 306)
40 µl water
If one sample produced a band, but the other did not:
2.5 µl PCR sample with band (locus 747)
5 µl PCR sample without band (locus 306)
42.5 µl water
If both samples produced bands on gel:
2.5 µl PCR sample with band (locus 747)
2.5 µl PCR sample (locus 306)
45 µl water

Dilutions For 3 loci:
*see the above chart for multiplexing primer pairs and filter info.

Total volume in each well of the 96 well plate should be 50µl.
If all samples produced no band on the gel:
5 µl PCR sample 1 (locus 747)
5 µl PCR sample 2 (locus 306)
5 µl PCR sample 3 (locus 839)
35 µl water
If one sample produced a band, but the others did not:
2.5 µl PCR sample with band (locus 747)
5 µl PCR sample without band 1 (locus 306)
5 µl PCR sample without band 2 (locus 839)
37.5 µl water

If two samples produced bands on gel, but one did not:
2.5 µl PCR sample with band 1 (locus 747)
2.5 µl PCR sample with band 2 (locus 306)
5 µl PCR sample without band (locus 839)
40 µl water

If all samples produced bands on gel
2.5 µl PCR sample (locus 747)
2.5 µl PCR sample (locus 306)
2.5 µl PCR sample (locus 839)
42.5 µl water

**96 well plate form: UVM Cancer Facility**

1. enter sample name and dilution on 96-well plate form
   - for single reaction, put 2X to indicate 90 µl water rather than 45
   - for multiplex, color code amount of water needed:
     * example 2 loci: red= 45 µl water; 2.5 µl of each PCR product
       green= 42.5 µl water; 2.5 µl 747, 5 µl 306
       purple= 40 µl water; 5 µl each sample
     - for multiplex, write locus and then either 2.5 or 5 to indicate amt. of PCR sample needed.

- Put water into 96 well plate.
- Put sample in appropriate well
- Seal with foil cover
- Put tape with excel file name on it over the last row of plate.

- Fill out excel form in folder marked “genescan order forms” – file is labeled import genescan. Label with your initials and the date (example: AMVZ011908)

- Bring plate to the Cancer Facility. Place on top, left shelf in Fragment Analysis fridge.

**Uploading Excel spreadsheet onto Biodesktop for Cancer Facility:**

1. login: avardo vardoschall
2. DNA depot
3. Fill out genescan form
4. Fill out name, phone number, budget, # samples, investigator.
5. Choose microsatellite markers
6. choose “yes” we have our own copy of genemapper  
7. Choose “No” to the multiplex option (even if you are- we have everything mixed into one well so they don’t need to know this)  
8. Choose 500bp size standard  
9. go to bottom of page and look for the option to upload (search) for a file. Click Browse  
10. locate the excel file and choose it to be attached.  
11. Hit the OK button.

**Getting files off of Biodesktop**

1. sign in  
2. DNA Depot  
3. click the new files you’d like to download.  
4. save to folder- I always save to my anne’s biodesktop folder and save the file with initials and the date (amv40706)  
5. logout.  
6. Unzip the file and choose a different folder to unzip to. Ahead of time, make a folder on the desktop with your initials and the date (jjs40706). This folder can also be within a larger folder (Summer 2006).  
7. Open Genemapper: username gm; password Plasmo  
8. add samples to project: find samples, add.  
9. choose “Anne’s data” for table format; “Microsatellite default” for Analysis method-copy down (highlight column, the n press ctrl D)  
10. Hit the Green Analysis button  

11. Name project with date and quick description (Cornell plate 6 2/2/08)  
12. For any red stop sign in the SQ (size quality) box, must examine size standard quality.  
   a. select sample  
   b. at top of the screen, click the ‘red peaks’  
   c. Do the peaks have labels (basepairs)? Should be YES  
      If YES: hit “override SQ” then ”OK”

7. Reanalyze samples by pressing the green analysis button again.  
8. Repeat steps 5-7 until all are “ok”

---

**Cornell Microsatellite Analysis**

Size Standard preferred: 600-Liz  
Dyes accepted: 6-FAM, NED, VIC, PET  
NO HEX OR ROX!
SAMPLE DILUTIONS:
Band on gel: 5ul PCR + 90ul H2O
No Band on gel: 5ul PCR + 45ul H2O

Making 96-well Plates:

Use ABI Specific 96-well plates only: Part # N801-0560; use strip caps to seal plate rows

In Freezer: HiDi from ABI. When you first get it, aliquot into 1.5 ml vials (fill with 1.5 ml). Freeze/thaw is bad!
- Put 1.5 ml HiDi into a 2.0 ml vial

In Fridge: 600-LIZ size standard alliquoted into 20 ul samples.
- Put 20 ul into HiDi 2ml vial.

Vortex like crazy. Vortex well between each row to keep size standard mixed evenly in the viscous HiDi.

- Into each well, 15.2 ul HiDi/Size standard mixture
  - Now add 1ul of your diluted PCR product.
    - Seal with strip caps
    - Label plate with tape on top and on side: Name and order #
    - Fill out online form to get order number

Sending: UPS next day padded envelope
  Wrap in paper towel
  See Joe for sample UPS form

Using Genemapper: Cornell Specific

1. Import files
2. Analysis Methods ➔ Microsatellite Default
3. Size Standard ➔ Cornell Better

4. Hit the Green Analysis button

5. Name project with date and quick description (Cornell plate 6 2/2/08)

6. For any red stop sign in the SQ (size quality) box, must examine size standard quality.
   a. select sample
b. at top of the screen, click the ‘red peaks’

c. Do the peaks have labels (basepairs)? Should be YES
   -If NO, need to create a brand new size standard entry specific for that sample

d. Peaks labeled ➔ Do they match up with the size standard sheet in the
   Genemapper notebook?
      1st peak= 1
      2nd peak= 20

   If YES: hit “override SQ” then ”OK”
   If NO,

      1. is the 1st peak labeled 20? Yes ➔ use the “-20 Cornell” size standard
      2. Is the 1st peak labeled 40? Yes ➔ use the “-40 Cornell” size standard

7. Reanalyze samples by pressing the green analysis button again.

8. Repeat steps 5-7 until all are “ok”

Recipes and Dilutions:

100 bp Ladder:
   450µl sterile H₂O to vial ➔ 2X solution
   Aliquot into two 1.5 vials. Freeze one.

Blue Juice Loading Dye:
   180 µl Dye + 180 µl sterile H₂O (add more H₂O until dye is thin)

Unlabeled Primers:
   Use excel program to determine amount of water needed for each primer
   Add specified amount ➔ 100µm solution
      10µl 100µm primer + 90 µl sterile H₂O ➔ 10µm primer

   Aliquot into 0.5 ml vials (12-20µl each). Freeze.
   For 2µm primers: 10µl of 10µm primer + 40µl sterile H₂O

Labeled Primers:
   100 µl sterile H₂O ➔ 100µm solution
   10µl 100µm primer + 90 µl sterile H₂O ➔ 10µm primer
   Aliquot into 0.5 ml vials (12-20µl each). Freeze.
1x TAE:
200 ml 25x TAE stock solution
4800 ml dH₂O

25x TAE:
1 packet of dry TAE 25X Ready-Pack buffer mix-- CAREFUL-- **wear a face mask**, do this outside! (Midsci Code 0912-2PK)

Slowly pour into a funnel, into a 1L Flask/Bottle half filled with 500ml dH₂O
Dissolve by gently swirling.
Add the final 500ml dH₂O

1% Agarose gel
3g Pure Agarose + 300ml 1x TAE
Heat until clear, but do not boil

Giemsa Buffer:
Acid: 9.07g KH₂PO₄ fill to 1000ml with dH₂O
Alkaline: 9.5g Na₂HPO₄ fill to 1000ml with dH₂O
Buffer: 61ml of Alkaline + 39ml of Acid +900ml dH₂O
Want pH of 7.0-7.2
Comprehensive Literature Cited


Babiker, H. A., A. M. Abdel-Muhsin, L. C. Randford-Cartwright, G. Satti, and D.


Dixon, PM. 1993. The bootstrap and the jackknife: describing the precision of


Eisen R.J. and N. M. Wright. 2001. Landscape features associated with infection by a malaria parasite (Plasmodium mexicanum) and the importance of multiple scale studies. Parasitology 122: 507-513.


Parasitological Research **102**: 571-576.


diversity and population structure of *Plasmodium falciparum* isolates from Dakar, Senegal, investigated from microsatellite and antigen determinant loci. Microbes and Infection **4**:685-692.


Nair, S., J. T. Williams, A. Brockman, L. Paiphun, M. Mayxay, P. N. Newton, J. P.


Perkins, S. L., S. M. Osgood, and J. J. Schall. 1998. Use of PCR for detection of


Roper, C., W. Richardson, I. M. Elhassen, H. Giha, L. Hviid, G. M. H. Satti, T. G.


Schall, J. J. 1990b. Virulence of lizard malaria: the evolutionary ecology of an ancient parasite host association. Parasitology 100:S35-S52


213


Waide, R. B., and D. P. Reagan. 1996. The rain forest setting. In The food web of a