Title: The drivers and consequences of unstable *Plasmodium* dynamics: A 26-year study of three malaria parasite species infecting a tropical lizard

Running title: Long-term drivers and consequences of parasite dynamics

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Abstract. The dynamics of vector-borne diseases are driven by interactions between factors intrinsic to the parasite, its host, and environmental fluctuations. Understanding these interactions requires a long-term view, especially to predict the consequences of climate change on parasite dynamics. We examined temporal fluctuations in risk of infection, its environmental drivers and consequences for host body condition of three malaria parasites (*Plasmodium azurophilum*, *P. leucocytica*, and *P. floridense*) infecting the lizard, *Anolis gundlachi*, in the rainforest of Puerto Rico. Our analyses included 13 surveys totaling > 8000 lizards over 26 years. During the early years of the study, prevalence of all three *Plasmodium* species appeared stable despite disturbances from short droughts and hurricanes. Taking the longer view, we found that the risk of infection and overall prevalence vary significantly over the years, and this fluctuation was driven in non-linear ways by variations in temperature and rainfall. We also found that risk of infection was maximized at intermediate levels of temperature and precipitation; relative proportions of the three *Plasmodium* species changed over time; and no significant negative effects of parasite infection on the lizard's body condition. Overall, the results emphasize the need for long-term studies to reveal host-parasite dynamics, their drivers and consequences.

Key-words*: Anolis*, body condition, climate change, long-term disease dynamics, malaria, parasite community, precipitation, Puerto Rico, temperature, virulence

INTRODUCTION

Understanding the drivers and consequences of infectious disease dynamics is a venerable challenge in ecology. From the first theoretical models developed in the 18th

century as part of smallpox control efforts to the Ross-Macdonald modeling approach that describes the dynamics of malaria prevalence (Smith et al. 2012), ecology has a long tradition studying long-term disease dynamics. Yet, how abiotic and biotic factors drive long-term disease risk and virulence remains an open question in disease ecology (Lively et al 2014).

Malaria parasites (*Plasmodium*) are vector-borne, and thus sensitive to environmental fluctuations. While malaria prevalence seems often stable and robust to environmental fluctuations, it can also change abruptly during epidemics (Schall et al. 2000, Chiyaka et al. 2013). Theoretical models predict stability, but only under the assumption of small environmental fluctuations; severe changes can push the system outside its stable equilibrium (Scheffer et al. 2001; Rohr et al. 2011). Thus, nonlinear links between prevalence and environmental terms can result in unstable dynamics. A current pressing issue is the possible influence of predicted climate change on malaria prevalence. Unfortunately, there is little consensus on the expected influence of climate change on the dynamics of vector-borne parasites including *Plasmodium* (Harvell 2009, Rohr et al. 2011).

Two connected issues are how changes in the dynamics of vector-borne parasites may be tempered both by the life history traits of the parasite itself (the "intrinsic factors" of epidemiology) and interactions among players in the overall parasite-host community. This story becomes circular if the parasite's prevalence drives evolutionary changes in both parasite and host life histories or changes the parasite community composition (Karvonen et al. 2013). Theoretical models suggest that transmission intensity plays into selection for parasite virulence (Ewald 1994, Schall 2002). These changes in virulence

can also mediate the outcome of competition among parasite species that share the same host including parasite colonization and/or extinctions, or changing individual species dominance within the community (e.g. Dobson and Pacala 1992, Karvonen et al. 2013).

Here we present a long-term study on the dynamics of three lizard malaria parasites (*Plasmodium azurophilum*, *P. floridense*, and *P. leucocytica*) infecting *Anolis gundlachi* in the tropical rainforest of Puerto Rico---a system that was undisturbed by deliberate human intervention. Schall et al. 2000 studied the long-term dynamics of this lizard-malaria system from 1990 to 1999 and found stable prevalence, no temporal variation in virulence and stable parasite community composition. Here we leverage their data (Schall et al. 2000), add extensive recent sampling, and reanalyze the data taking a longer-term view spanning 26 years to ask: (1) Is parasite prevalence and risk of infection stable during the 26 years' period? (2) If not, is this temporal variability driven by abiotic factors (e.g., temperature and precipitation)? (3) Was parasite species composition stable during this period? (4) Was there temporal variability in parasite virulence to the host?

METHODS

Study system, field sampling, and diagnostics

We sampled lizards in a rainforest at the El Verde Field Station at the Luquillo Experimental Forest in eastern Puerto Rico (N 18°19.263'- W 65°49.146'). *Anolis gundlachi* is among seven anoles at the site, but the others are only very rarely infected (Schall et al. 2000). We sampled during 13 periods over 26 years: summers (May-August) 1990, 1996, 1997, 1998, 2015, 2016, and winters (January-March) 1991, 1997,

1998, 1999, 2001, 2002 and 2016. To insure consistency over time we replicated rigorously the protocols of field sampling and diagnostics used in the original study by Schall et al. (2000). Specifically, we sampled the same sites within the forest (between 4 and 10 trails each year) and collected similar sample sizes ($N > 650$). Infection status was determined by examination of stained blood smears, and parasite species scored based on morphological traits and cell class infected (Telford 2016).

We compiled temperature and rainfall data from the El Verde weather station records and estimated the daily mean minimum, maximum, and variance of registered temperatures and rainfall 30 and 120 days prior to the sampling month (Schall et al. 2000). Hurricane events occurred in September 1995 (two), July 1996, and September 1998. We do not include hurricanes *per se* in the analysis, but high rainfall would mark those periods in the precipitation data. To estimate host body condition– a sub-lethal measure of parasite virulence–we measure the snout-vent length (SVL) and mass of each lizard. Detailed field and laboratory methods and collection locations are given in the Supplemental Information.

Analyses

To determine if the risk of infection was stable through the 26-year study period, we compared eight binomial models in their ability to predict the individual probability of infection. Each model represents a hypothesis describing what factors better explained temporal variability in infection probability. These models (with exception of the null model) included sex, season and/or SVL as a predictor because Schall et al. 2000 found strong evidence showing that the probability of infection increases with body size (SVL) with males having a higher probability of infection than females (sex). They also found

that the probability of infection was higher in the summer than in the winter (season). We compared models predicting the probability of infection as a function of (1) SVL, year, and sex separately, (2) the additive effect of sex, SVL and season, (3) a similar model with an additive effect of year (4) a model including the same sex, SVL and season with an interactive effect between sex and SVL, (5) a similar model with an additive effect of year, and (6) an intercept-only model that represented the null hypothesis that neither of the tested variables explains the observed temporal variability in probability of infection. These models were fitted using maximum likelihood and compared using Akaike Information Criterion adjusted for small sample sizes (AICc), where the most parsimonious model will result in the lowest score. If there was significant temporal variation in the dynamics, the most parsimonious model (lowest AICc score) would include the effect of sampling year.

We followed a similar model selection approach to test which environmental variables (i.e., rainfall and/or temperature) better predicted the proportion of infected individuals. Following Schall et al. 2000, we compared models that included mean rainfall 30 days and 120 days before the field sampling. We also fitted models with the variance of rainfall or temperature 30 or 120 days before the field sampling (Vasseur et al. 2014). To allow for a possible non-linear effect we also fitted individual and additive models with a second-degree polynomial. Last, we fitted a null (intercept only) model to describe the case where none of the tested variables better explains the observed patterns in prevalence. All models were fitted using maximum likelihood and we used AICc to select the most parsimonious model.

To assess changes in composition of the three *Plasmodium* species through time

we compared 10 multinomial logit models in their ability to predict the individual probability of being healthy, or infected by one of three *Plasmodium* parasites (four categories; co-infections were not considered). This modeling approach is an extension of a logistic regression for multinomial response variables. These models included (1) the single effect of sex, SVL, or year; (2) models considering the additive and interactive effect of year and SVL; (3) a model considering the additive effect of sex and SVL; (4) the additive effect of these three variables, with either interactions of year with SVL and sex. We also fitted a null (intercept-only) model that represents the case where any of these variables explains the probability of getting infected by either of the three *Plasmodium* parasites. The models were fitted using maximum likelihood using the nnet package and compared using AICc.

To estimate body condition (a proxy for sub-lethal parasite virulence to the host; Schall and Pearson 2000) we used the residual index R_i (Cox and Calsbeek 2015). We calculated this index using the residuals of the linear regression of log10 mass on log10 SVL. We made a separate analysis for each sex, and for each year for which body mass and SVL data were available (1996, 1997, 1998, 2015, and 2016). Lizards with positive residuals are heavier than average (better body condition), while lizards with negative residuals are skinnier relative to their SVL than average (Schall et al. 2000). To test for differences in body condition due to infection state or time, we used a two-way ANOVA, where the two factors were year and infection state (infected vs. non-infected). All analyses were done in R 3.0.3 (R Development Core Team 2016).

RESULTS

Long-term dynamics and its environmental drivers

A total of 8055 *Anolis gundlachi* lizards were sampled over the 26-year period. The most parsimonious model explaining the variability in the risk of infection in this period included the additive effect of sex, SVL, season, year, and the interaction between sex and SVL (ΔAIC = 8.58 between this model and the next best; Table S1). For both summer and winter samples, the risk of infection was relatively stable for the early years, dropping significantly during the 2015–2016 period (Fig. 1, Table S2). Overall prevalence dropped ~ half for males and > half for females from 1990–2002 vs. 2015–2016 (Fig. S1).

The most parsimonious model explaining temporal variations in the proportion of infected individuals included the additive quadratic effect of mean maximum daily temperature and mean daily rainfall through 120 days before the sampling (ΔAIC = 90.37 between this model and the next best; Table S3). The risk of infection followed a nonlinear response to temperature at a variety of rainfall levels (Fig. 2, Table S4). Maximal predicted risk of infection was maximized at a minimum temperature of ~ 26° and rainfall of 9.62 mm.

Long-term changes in parasite species composition

The most parsimonious model explaining the probability of an individual being healthy, or infected by *P. azurophilum*, *P. leucocytica* or *P. floridense* included the additive effect of SVL, sex and year (ΔAIC = 3.29 between this model and the next best; Table S5). This model predicts a decrease in the probability of infection of all *Plasmodium* parasites after 2002 (Fig. 3). Thus, the drop in prevalence during the most recent sample periods was not due to only one species of parasite declining, but an overall decline. *P. azurophilum* was consistently the most common species, and remained

at a similar proportion of all infections throughout the 26-year period. *P. floridense* and *P. leucocytica* changed their relative dominance, but this apparently was due to a secular decline in *P. floridense* over the entire study period. This model predicted no differences in the partial relationship between SVL and probability of infection by the different *Plasmodium* parasites (Fig. S4). Whereas the model predicts little differences between sexes in the probability of getting infected by *P. azurophilum*, the probability of getting infected by *P. floridense* and *P. leucocytica* is predicted to be greater in females (Fig. S5).

Long-term changes in the body condition due to infection

The body condition index (BCI) of females in the summer did not vary significantly by infection state ($F_{1,753}$ = 3.84, P = 0.05), year ($F_{3,753}$ = 0.039, P = 0.99) or their interaction ($F_{3,753}$ = 0.56, P = 0.64; Fig. S6). We found a similar pattern in males in the summer season where BCI did not vary significantly by infection state ($F_{1,1357}$ = 0.042, P = 0.84), year ($F_{3,753}$ = 0.001, P = 1.0) or their interaction ($F_{3,753}$ = 0.530, P = 0.66; Fig. S6). The pattern in the winter season resembles that of the summer where BCI for females did not vary significantly by infection state ($F_{1,356}$ = 3.01, P = 0.084), year ($F_{2,356}$ = 0.12, P = 0.89), or their interaction ($F_{1,356}$ = 1.36, P = 0.24; Fig. S6). BCI for males in the winter did not vary significantly by infection state ($F_{1,752}$ = 0.42, P = 0.52), year ($F_{2,752}$ = 0.01, P = 0.99), or their interaction ($F_{2,752}$ = 0.36, P = 0.70; Fig. S6).

DISCUSSION

We assembled a long-term data set spanning 26 years to explore the dynamics of three malaria parasites and their vertebrate host. This is one of the longest of such studies on a

natural system of a vector-borne disease infecting a nonhuman host (see also Schall and St. Denis 2010). Our results show that risk of infection for the anole by *Plasmodium* parasites varied little early in the study despite several short droughts and hurricane events. Prevalence of all three *Plasmodium* species, though, declined substantially during the most recent years. Overall, variation in risk of infection was associated with fluctuations in temperature and rainfall four months before each sample period, but the relationship with temperature was nonlinear such that maximum risk of infection is predicted at an intermediate temperature and rainfall. The recent drop in risk of infection coincided with a severe drought in Puerto Rico. During the study, one species, *P. azurophillum* was consistently the most common parasite, but one of the others, *P. floridense*, declined slowly. Finally, we found that body condition did not fluctuate significantly over time for both summer and winter periods and was not significantly different between non-infected and infected individuals. Overall, these patterns were not apparent during the early years of the study; thus, a long-term approach was needed to reveal the true response of the parasite dynamics to environmental changes.

The delayed link between rainfall and temperature with parasite prevalence (120 days) most likely is a consequence of shifting vector abundance and biting behavior. Unfortunately, the identity of vector(s) of the three parasite species is not known, but *P. floridense* is known to infect *Culex* mosquitoes in Florida (Klein et al. 1987). During the severe 2015 drought we noted an overall dry aspect to the forest, with little standing water in puddles, *Heliconia* flowers or epiphytic bromeliads, which could have restricted breeding locations for mosquitoes. Most striking, despite the general effect of variation in temperature and humidity over the 26-year period, no dramatic effect on prevalence,

nor risk of infection, was seen after short environment disruptions from dry periods or severe hurricanes during the first 10 years of the study period. Schall et al. (2000) noted that the hurricanes caused severe physical damage to the forest, including massive defoliation. Thus, the drop in risk of infection in the later samples could indicate that the 2015 drought was of sufficient duration to shift the system to a new dynamic state.

But, what caused the differences in parasite prevalence for the summer vs. winter seasons? Rainfall is greater in the summer, which increases potential habitats for vector breeding increasing the rate of vectors to hosts. Also, summer is the mating season for *A. gundlanchi* anoles when lizards are more active and defending territories (Reagan 1992, Gorman and Licht 1974). If the vectors are daytime active, the lizards could be more exposed to vector bites. Alternatively, the lower prevalence in winter may be only apparent if the parasite reduces reproduction in the lizard blood if vector activity ceases. Using a PCR-based method to detect very low-parasitemia infections and cryptic infections were more common in the winter season (unpublished data).

Our study adds to the growing evidence that the relation between prevalence of malaria parasites and climatic measures is complex, and often nonlinear (Mordecai et al. 2013, Garamszegi 2011). Adding to the complexity is the role of spatial scale (Wilkinson et al. 2016). For example, in the *Sceloporus occidentalis*- *Plamodium mexicanum* system in California, when looking at the relationship between landscape features and infection prevalence, the type of ground cover (grass, rocks, and leaf litter) affected the probability of capturing infected or healthy lizards (Eisen and Wright, 2001). Note that changes in temperature and rainfall could alter microhabitat quality, such as production of leaf litter and ground cover. Thus, suggestions that simple rising temperatures during climate

change will increase prevalence of malaria parasites ignore the more complex story. Indeed, our data suggest anole's risk of infection by malaria parasites may decrease in current climate change scenarios.

The El Verde lizard malaria system has an added interest because we could follow through time the relative proportions of three species of *Plasmodium* that infect a single lizard host. Despite the major drop in the probability of infection during the later sample periods, and the major disruption of the forest by hurricanes in the early years, *P. azurophilum* remained at a constant higher proportion of infections. *P. floridense* gradually changed over the years and switched proportion of infections with the third species, *P. leucocytica*. What could account for this secular change? These two species infected different cell classes (erythrocytes for *P. floridense* and several classes of white blood cells for *P. leucocytica*), and therefore are not likely to be competitors for within-host habitats, and are unlikely to cross-react with the immune system. The competitive interactions of malaria parasite species that exploit the same host are poorly studied (but see Schall and Bromwich, 1994). We suspect the explanation falls to the biology of the vectors, either different insect host species or how parasites may compete within a single vector.

Body condition is used as a proxy of the energetic state of lizards and their fitness (Cox and Calsbeek 2015). Our results are consistent with previous studies that found little evidence of a relationship between lizard health and parasite infection in the Caribbean (Schall and Pearson 2000; Schall and Staats 2002). This may be explained by two non-multually exclusive explanations. First, parasitaemia of Caribbean *Plasmodium* species is lower than those of California or Africa, which may result in a lower rate of

blood cells turnover. Second, low virulence could be the result of lower transmission intensity as predicted by the theory of virulence evolution (e.g., Ewald 1994).

We conclude that the most significant finding of this study is the value, even the necessity, of a long-term approach (> 20 yrs) to understanding the ecology of host-parasite systems. Studying the *A. gundlachi* - *Plasmodium* spp. system over decades allowed us to gain a better picture of the patterns and possible mechanisms behind prevalence changes, shifts in the blood parasite community, and the effects on the body condition of the hosts. The influence of environmental variables, exactly the kind expected with ongoing climate change, became apparent only after taking this long-term approach.

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LITERATURE CITED

Chiyaka, C., A. Tatem, J. Cohen, P. Gething, G. Johnston, R. Gosling, R. Laxminarayan, S. Hay, and D. Smith. 2013. The stability of malaria elimination. Science 339:909-910.

Cox, R. M., and R. Calsbeek. 2015. Survival of the fattest? Indices of body condition do not predict viability in the brown anole (*Anolis sagrei*). Functional Ecology 29:404-413.

Dobson, A. P., and S. W. Pacala. 1992. The parasites of *Anolis* lizards the northern Lesser Antilles. II. The structure of the parasite community. Oecologia 92:118–125.

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

Ewald, P.W. 1994. The Evolution of Infectious Diseases. Oxford University Press, Oxford, U.K.

GaramszegI, L. Z. 2011. Climate change increases the risk of malaria in birds. Global Change Biology 17:1751–1759.

Gorman, G. C., and P. Licht. 1974. Seasonality in ovarian cycles among tropical *Anolis* lizards. Ecology 55:360-369.

Harvell, D., S. Altizer, I. M. Cattadori, L. Harrington, and E. Weil. 2009. Climate change and wildlife diseases: when does the host matter the most? Ecology 90:912–920.

Karvonen, A., B. K. Kristjánsson, S. Skúlason, M. Lanki, C. Rellstab, and J. Jokela. 2013. Water temperature, not fish morph, determines parasite infections of sympatric Icelandic threespine sticklebacks (*Gasterosteus aculeatus*). Ecology and Evolution 3:1507–1517.

Klein, T. A., Young, D. G., & Telford Jr, S. R. (1987). Vector incrimination and experimental transmission of *Plasmodium floridense* by bites of infected *Culex*

(Melanoconion) *erraticus*. Journal of the American Mosquito Control Association, *3*(2), 165-175.

Lively, C. M., J. C. de Roode, M. A. Duffy, A. L. Graham, and B. Koskella. 2014. Interesting open questions in disease ecology and evolution. American Naturalist 184: S1–S8.

Mordecai, E. A., K. P. Paaijmans, L. R. Johnson, C. Balzer, T. Ben□Horin, E. Moor, A. McNally, S. Pawar, S. J. Ryan, and T. C. Smith. 2013. Optimal temperature for malaria transmission is dramatically lower than previously predicted. Ecology letters 16:22–30.

R Core Team. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://r-project.org

Reagan, D. P. 1992. Congeneric speies distribution and abundance in a three-dimensional habitat: the rain forest anoles of Puerto Rico. Copeia 1992:392-403.

Rohr, J. R., A. P. Dobson, P. T. Johnson, A. M. Kilpatrick, S. H. Paull, T. R. Raffel, D. Ruiz- Moreno, and M. B. Thomas. 2011. Frontiers in climate change-disease research. Trends in Ecology and Evolution 26:270–277.

Schall, J. J. and K. M. St. Denis. 2010. Lack of sequence variation of the mitochondrial cytochrome b gene from a malaria parasite, *Plasmodium mexicanum*. Journal of Parasitology 94:827-829.

Schall, J. J. and C. M. Staats. 2002. The virulence of lizard malaria: Three species of *Plasmodium* infecting *Anolis sabanus*, the endemic Caribbean anole of Saba, Netherlands Antilles. Copeia 2002:39-43.

Schall, J. J. (2002). Parasite Virulence 1 4. The behavioural ecology of parasites, 283.

Schall, J. J., A. R. Pearson. 2000. Body condition of a Puerto Rican Anole, *Anolis gundlachi*: Effect of a malaria parasite and weather variation. Journal of Herpetology 34: 489–491.

Schall, J., A. R. Pearson, and S. L. Perkins. 2000. Prevalence of malaria parasites (*Plasmodium floridense* and *Plasmodium azurophilum*) infecting a Puerto Rican lizard (*Anolis gundlachi*): a nine-year study. Journal of Parasitology 86:511–515.

Schall, J. J. and C. R. Bromwich. 1994. Interspecific interactions tested: Two species of malarial parasite in a west African lizard. Oecologia 97:326-332.

Scheffer, M., S., Carpenter, J. A., Foley, C., Folke, and B., Walker. 2001. Catastrophic shifts in ecosystems. Nature 413:591–596.

Smith, D. L., K. E. Battle, S. I. Hay, C. M. Barker, T. W. Scott, and F. E. McKenzie. 2012. Ross, Macdonald, and a theory for the dynamics and control of mosquito-transmitted pathogens. PLoS pathogens 8:e1002588.

Telford Jr, S. R. 2016. Hemoparasites of the Reptilia: Color atlas and text. CRC Press.

Vasseur, D. A., J. P. DeLong, B. Gilbert, H. S. Greig, C. D. Harley, K. S. McCann, V. Savage, T. D. Tunney, M. I. O'Connor. 2014. Increased temperature variation poses a greater risk to species than climate warming. Proceedings of the Royal Society of London B: Biological Sciences 281: 20132612.

Wilkinson, L. C., C. M. Handel, C. Van Hemert, C. Loiseau, and R. N. Sehgal. 2016. Avian malaria in a boreal resident species: long-term temporal variability, and increased prevalence in birds with avian keratin disorder. International journal for parasitology 46:281–290.

Figure captions:

Fig 1. Risk of infection of *Anolis gundlachi* by three malaria parasite species over time at a rainforest in eastern Puerto Rico. Risk of infection is higher in the summer vs. winter months. Note how the risk of infection is relatively constant in the early years, but decreases in 2015–2016. Dots are the infection probability estimates and bars represent the standard errors.

Fig 2. Predictions of the most parsimonious model explaining the temporal variation in the proportion of infected *Anolis gundlachi* by malaria parasites. This model predicted the proportion of infected individuals by the additive effect of squared mean daily rainfall and mean daily temperature 120 days before sampling. Lines represent estimated means and shaded areas 95% confidence intervals.

Fig 3. Partial predictions from the most parsimonious multinomial model showing a general decrease through time in the risk of infection of *Anolis gundlachi* by three *Plasmodium* parasites. The model predicts a decrease in the probability of infection by *P. floridense* in the last three censuses compared to *P. leucocytica*. Dots represent the infection probability estimates by each *Plasmodium* species and the bars represent 95% confidence intervals. The letters “w” and “s” following the year describes the winter of summer season respectively.

Figure 1.

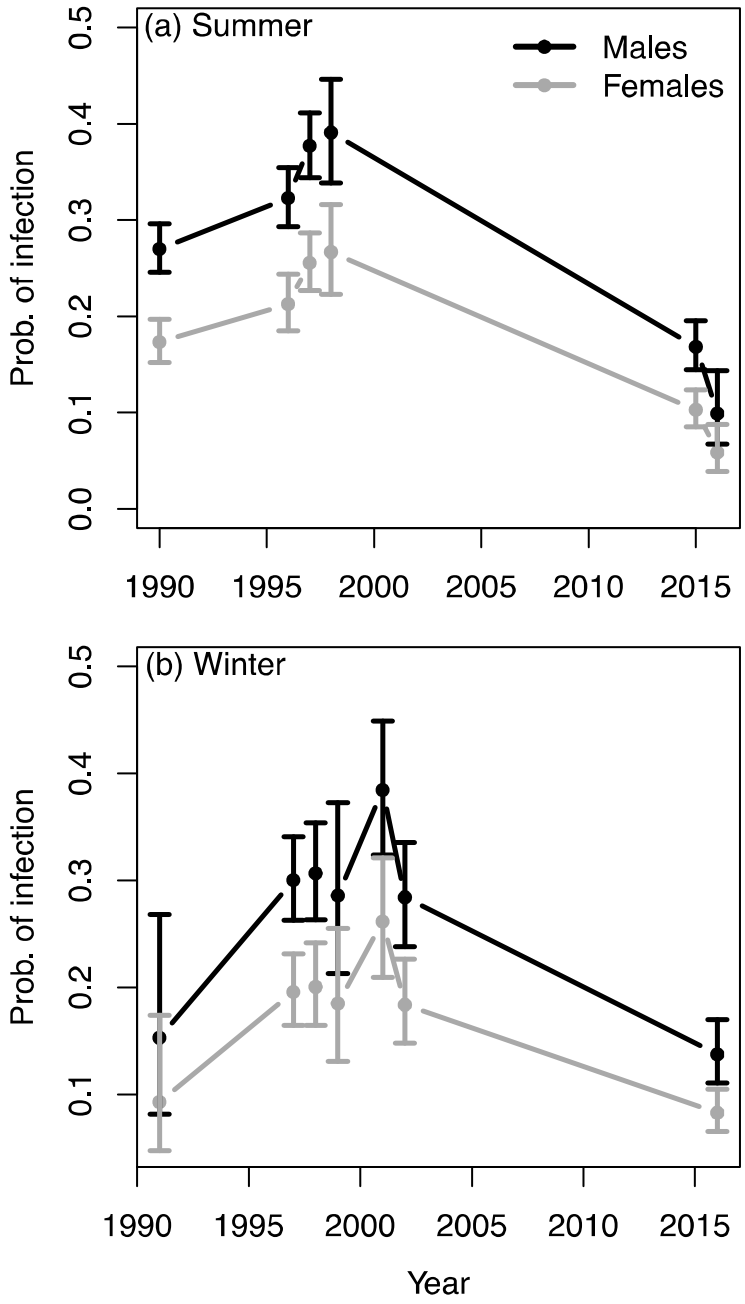

Figure 2

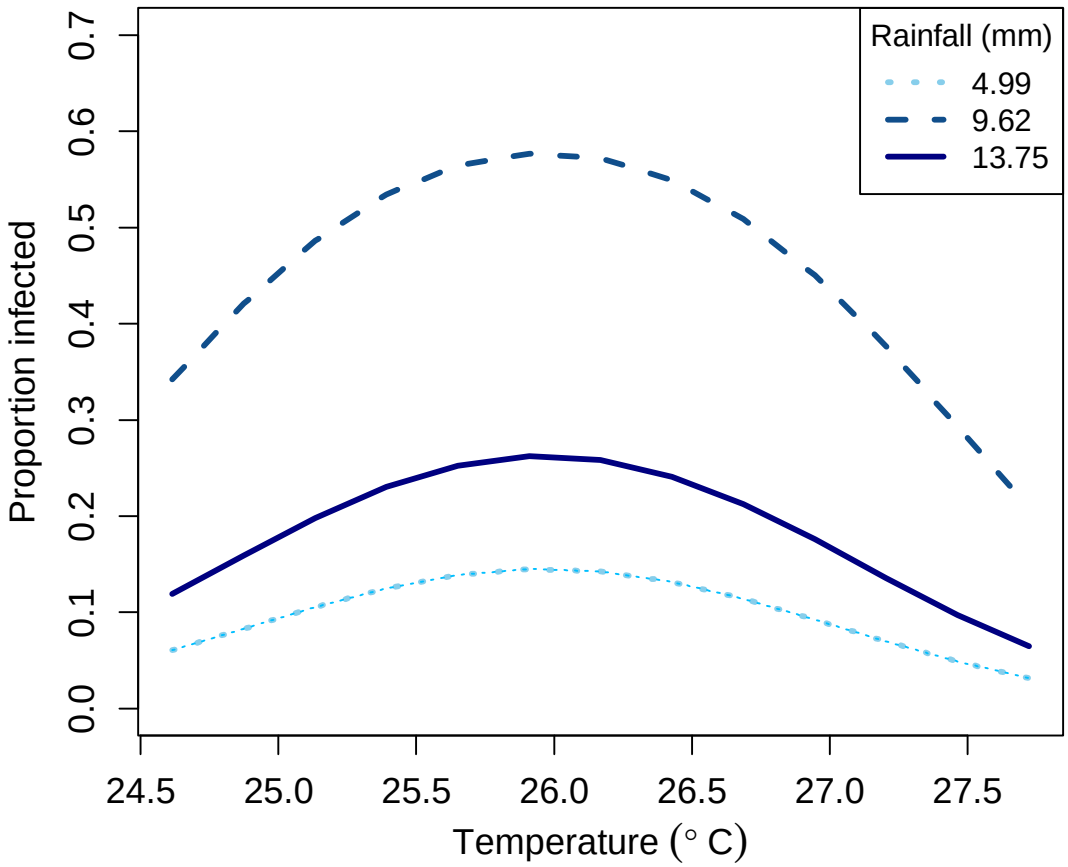

Figure 3