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Mosquito Prevalence and Malaria Risk in Relation to Land-Use Near Ranomafana National Park in Madagascar

By

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Master of Public Health

Global Environmental Health

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# Mosquito Prevalence and Malaria Risk in Relation to Land-Use Near Ranomafana National Park in Madagascar

By

Kristin Derfus

B.S.

University of Florida

2011

Thesis Committee Chair: Dr. Thomas Gillespie, Ph.D.

An abstract of

A thesis submitted to the Faculty of the
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2014

## Abstract

Mosquito Prevalence and Malaria Risk in Relation to Land-Use Near Ranomafana National Park in Madagascar
By Kristin Derfus

*OBJECTIVE.* To examine the association between mosquito prevalence and malaria risk in varying land-use sites in and around Ranomafana National Park in Madagascar.

*METHODS.* Analyses were based on mosquitoes trapped in six different villages and the surrounding forests and agricultural sites from June to August of 2013. Two CDC miniature light traps were set in each land-use site and trapping took place for a minimum of three nights in each of the 6 different village, forest and agricultural locations. Trapped mosquitoes were collected, desiccated and sent to the CDC for analysis. ELISA was used to test all female *Anopheles* mosquitoes for the malaria parasite, *Plasmodium*. PCR was used to confirm positive ELISA results.

*RESULTS.* A total of 2,033 mosquitoes, with 415 *Anopheles,* were trapped during the 21 nights of trapping. Based on Poisson regression analysis, agricultural land-use sites had a higher prevalence of *Anopheles* mosquitoes than the forest and village sites. Interestingly, forest sites had the lowest prevalence of *Anopheles*. Close proximity to animal pens was also associated with higher prevalence of *Anopheles* mosquitoes. Three mosquitoes were ELISA-positive, but PCR results did not confirm the presence of *Plasmodium* DNA.

*CONCLUSION.* Data demonstrates that agricultural sites have a higher prevalence of *Anopheles* mosquitoes than forest or village sites. In addition, proximity to domesticated animal pens was associated with higher prevalence of *Anopheles*, but sample size precluded detailed examination of this relationship. Further investigation will be required to develop specific predictions of risks for malaria transmission and potential for malaria interventions.

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In addition, my laboratory analysis would not have been possible without the training and supplies provided by the Centers for Disease Control and Prevention, Division of Parasitic Diseases and Malaria. And my data analysis was greatly improved with the help of Dr. Devine and Emory Biostatistics graduate student, Jiaxiang Gai.

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# Introduction

## *Malaria*

Malaria is caused by the parasite, *Plasmodium* and is transmitted by the mosquitoes of the genus *Anopheles*. There are approximately 430 *Anopheline* mosquito species, but only 30-40 of them are capable of transmitting malaria (CDC, n.d.). There are five species of *Plasmodium* that cause malaria in humans: *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi* (CDC, n.d.). The two most common species of public health interest are *P. falciparum* and *P. vivax* (WHO, n.d.). Humans can act as a reservoir for the parasite and the mosquitoes are the vectors for the parasite transmission. During infection of a human, malaria can cause a spectrum of symptoms from uncomplicated (i.e., fever) to severe (i.e., anemia and death) (WHO, n.d.). The different species of *Plasmodium* cause distinctly different symptoms, with *P. vivax* uniquely causing relapse infections (Craig & Kain, 1996). *Plasmodium vivax* also has different strains that vary in symptoms (Craig & Kain, 1996).

## *Malaria Around the World*

About half of the world's population is at risk for malaria and there are an estimated 627,000 annual malaria deaths globally ("Global malaria fight," 2013). Although the current prevention and treatment methods have greatly reduced morbidity and mortality caused by malaria, limited access to these interventions has cost many lives in the rural tropics. Available funding for malaria prevention in 2012 was approximately $2.5 billion, which was only half of the funds necessary to provide prevention and treatment resources globally ("Global malaria fight," 2013). If we assume a constant level

of funding for interventions, malaria control programs must start using these limited available resources more efficiently. Understanding environmental effects on malaria vectors and transmission risk has the potential to help predict the location of transmission hotspots to be targeted for preventative measures.

### *Malaria in Madagascar*

The government of Madagascar has dramatically increased both internal and external financing to decrease malaria infection rates. Despite this, malaria is endemic in 90% of the country, with the entire population considered at risk (PMI, n.d.). The area surrounding the protected Ranomafana National Park, in southeastern Madagascar, has perennial transmission of malaria (PMI, n.d.). This is in stark contrast to the lowland areas in the northwest that have specific transmission seasons, and the highland (central) and semi-desert areas (southwest) areas of the country that have unstable seasonal transmission (PMI, n.d.).

### *Land-Use Patterns and Infectious Disease*

Deforestation has been linked to deleterious effects on environmental conditions capable of enhancing opportunities for human pathogens (Patz et al, 2000; Pongsiri et al, 2009). In addition, certain agricultural practices may alter the nutrient content of soil, as well as watershed dynamics, in ways that increase breeding of mosquitoes (Vittor, 2009). For example, irrigation can cause an increase in the number of mosquitoes, which sometimes leads to an increase in malaria prevalence (Mutero et al., 2006).

In Madagascar, multi-generational unsustainable agricultural practices (i.e., slash and burn monoculture) in association with human population growth has led to rapid

forest conversion and the use of even the least productive lands for rice production (Sussman, 1994).

### *Targeting Environmental Factors in Malaria Control*

Although increased financing for malaria treatment and mosquito control with insecticides has successfully decreased malaria rates in Madagascar, addressing environmental determinants of disease could provide additional ways of controlling malaria that would increase the efficiency of programs (Utzinger, 2001). The environmental aspect of malaria control has generally incorporated environmental management practices such as vegetation clearance, draining swamps and modification of river boundaries (Mutero, 2005) (Utzinger, 2001). Possibly a less invasive control system could be the analysis of key environmental determinants of disease to guide revisions to current practices by identifying land-use patterns associated with higher or lower risk (Mutero, 2005) (Utzinger, 2001).

### *Parasite/Mosquito Species*

The species of *Anopheles* mosquitoes, as well as the species of *Plasmodium,* are important factors to take into account when evaluating the threat of malaria in different regions. For *Anopheles,* the particular species can determine whether the mosquito is zoophilic, anthropophilic or simply an opportunistic feeder (Dekker & Takken, 1998). With this information, we can evaluate the risk that a particular population of mosquitoes represents. In addition, the species of *Plasmodium* also matters in terms of severity of disease and the risk for morbidity and mortality (Ewald, 1983).

According to the WHO 2013 World Malaria Report for Madagascar, the expected species of *Anopheles* in the region are *An. funestus, An. gambiae,* and *An. arabiensis*

("World malaria report," 2012). Of the five *Plasmodium* species capable of infecting humans with malaria, four of them are present in Madagascar (Barnadas, 2008). *Plasmodium falciparum* is the most prevalent species in Madagascar, with *P. vivax* being the second most prevalent (Barnadas, 2008). However, *P. vivax* is understudied in comparison to *P. falciparum* (Barnadas, 2008).

### *Importance of Studying Malaria in Madagascar*

Madagascar is a unique country because of its isolated environment, with distinctly varying landscapes and a rich diversity of animals. However, one of the most distinct characteristics of Madagascar is the lineage of its human inhabitants, which are of both Austronesian and African descent (Menard et al., 2010). This diversity of the human population has resulted in a mixture of certain genetic characteristics of Austronesian and African people. One of those genetic characteristics is the Duffy negative blood group that is largely of African ancestry (Menard et al., 2010). Duffy blood group negative people have been shown to be resistant to the erythrocyte *P. vivax* infection (Menard et al., 2010).

Madagascar is composed of a heterogeneous population with and without the Duffy blood group (Menard et al., 2010). Studies within the country have shown that *P. vivax* is no longer dependent on the Duffy antigen for establishing human blood-stage infection and disease (Menard et al., 2010). Thus, *P. vivax* has found a different way to enter the blood cell. This is a phenomenon that has only been found to occur in Madagascar, but has the potential to have major impact on malaria control efforts around the world, especially in regions with Duffy-negative people, such as West, Central and Southern Africa ("Duffy-negative blood," 2010; Menard et al., 2010). A potential reason

for the increases in Duffy-negative susceptibility to *P. vivax* may be population mixing in Madagascar ("Duffy-negative blood," 2010; Menard et al., 2010). Overall, whether this change is due to the mixture of ancestry in Madagascar or something unrelated, Madagascar appears to be in a unique situation that can put its people at increased risk for malaria.

## Objectives

This project incorporated field and laboratory methods to examine how land-use affects the abundance of malaria vector and parasite prevalence. Malaria transmission in this part of Madagascar is perennial with clear seasonality (Roca-Feltrer et al., 2009). Mosquito collection took place during a season of lower transmission. By identifying the few populations of mosquitoes where the malaria parasite, *Plasmodium*, was maintained during the dry season, foci for disease risk were identified. Such geographic data on vectors and parasites can inform and greatly improve malaria control and elimination programs (Mendis, 2009).

The goal of this project was to determine the effect of land-use on the prevalence of *Anopheles* mosquitoes and the effect of land-use on the proportion of *Anopheles* mosquitoes infected with *Plasmodium* in rural Madagascar. These data may prove valuable to improve our estimates of the potential risk of human infection with malaria in relation to varying land-use in this biodiversity hotspot. I predicted that forest cover would be inversely related to, and agricultural intensity would be positively associated with, *Anopheles* density, as these aspects of land-use strongly affect the availability and quality of breeding sites for *Anopheles* mosquitoes. Areas with decreased forest cover and increased agricultural intensity would in turn increase *Anopheles* density in the

surrounding area. If the *Anopheles* are in close enough proximity to *Plasmodium* infected humans in order to feed, then *Anopheline* population increase would be directly associated with increased *Plasmodium* transmission and an increased risk of malaria in the area.

# Materials and Methods

## Ethics Statement

All research protocols were presented to and approved by the USDA and the Government of Madagascar to gain the necessary permits for research and importation of mosquitoes. The United States Veterinary Permit for Importation and Transportation of Controlled Materials and Organisms and Vectors (Permit #: 107234) was the authorization form obtained for the imports.

## IRB

This specific study does not involve human subjects and does not require IRB approval.

## Site Selection

The study site was in and around the Ranomafana National Park (21°02'–21°25'S, 47°18'–47°37'E), which is in the remaining southeastern rainforest of Madagascar, lying about 144 kilometers west of the Indian Ocean ("TEAM," n.d.). The Ranomafana National Park is a continuous humid tropical forest with natural vegetation ranging from montane cloud rainforest to lowland rainforest following an altitudinal gradient from 1,513 to 600 meters (Wright, 1997). Temperature varies from 3 to 30°C ("TEAM," n.d.).

The climate of Ranomafana includes a prolonged monsoon season (January to April) with average annual rainfall ranging between 2500 and 4000 mm ("TEAM," n.d.).

Trapping took place in six villages and in agricultural sites and forest sites associated with each village. In most cases, each of the land-use sites are situated right next to each other, without any type of buffer zone (Figure 3). Forest sites included primary and secondary forest within national park boundaries. These sites had zero human inhabitants and ranged from little (forest trails, trails to a few homes, etc.) to zero daily human overlap (Figure 1.A). Villages were defined as communities with at least 10 homes within 15 meters or less from one another. The villages chosen as testing sites had at least 30 people living within the main village perimeter and the traps were set within this perimeter (Figure 1.B). Agricultural sites mainly consisted of rice paddies, but also included areas with vegetable gardens and banana trees (Figure 1.C). The coordinates of each trapping location were determined using a Garmin GPS, and the coordinates were recorded (Table 1).

Larval dipping was also done in each of the six villages and surrounding agricultural and forest sites. However, it is important to note that larval dipping was not performed at each trapping site specifically.

## Collection Method

### *Trapping*

Mosquitos were collected from 17 June to 6 August 2013, using a CDC miniature light trap (Model 512, John W. Hock Company) and $CO_2$ (Figure 2). The $CO_2$ was produced by fermentation of brown sugar and yeast in warm water. Half of the traps at each land-use site were also baited with a human odor, 3-Methyl-1-butanol (Mukabana,

2012). Each trap had a light sensor (LCS-2 PhotoSwitch, part number 1.60, John W. Hock Company) attached that triggered the fan and light to turn on at sunset and off at sunrise, ensuring that trapping was consistently beginning and ending at the same levels of light during each trapping session. Trapping at each location took place for a maximum of 3-4 consecutive nights, due to logistical constraints. Traps were powered by 6-12 volt batteries recharged after each 4-night session.

### *Larval Dipping*

Larva/pupa were collected at each land-use site, for general count data only. A larval dipper was constructed using a measuring cup fixed to the end of a broomstick. At each land-use site (forest, village, agriculture) sitting pools of water were sought out in order to search for larva. In each village, the total search area was minimal and the amount of sitting water was also minimal and sometimes non-existent, so the entire village was searched. However, due to the vast areas that the forest and agricultural sites covered, only paths between the two traps were searched for sitting water. Three dips were sampled from each water source. Due to uncertainties in morphological characteristics of the larva/pupa of each mosquito genus, counts were recorded if there was simply confirmation of mosquito larva/pupa.

### *Sorting & Preserving*

Adult mosquitoes (larva/pupae were not saved) were identified morphologically to genus using the CDC Environmental Health Service "Pictorial Key to Mosquitoes," in addition to utilizing identification assistance from a Malagasy Entomology student at the University of Antananarivo, Tovo Mbolatiana Andrianjafy ("Pictorial Key"). All mosquitoes and *Anopheles* were counted to determine prevalence. Adult *Anopheles*

mosquitoes were put in specific vials based on trap night and site. The remaining mosquitoes, which were mainly *Culex*, were also kept and labeled based on trap night and site. Mosquitoes were preserved in vials using Drierite (Fisher Scientific catalog #: 075783B) and brought to Emory University, Atlanta, Georgia, USA.

## Laboratory Analysis

### ELISA

All collected female *Anopheline* mosquitoes were subject to Enzyme-linked immunosorbent assays (ELISA). The mosquitoes were dissected into head/thorax and abdomen portions. The head and thorax portion was crushed in a buffer and used for the ELISA and the abdomens of each *Anopheline* mosquito were saved for additional PCR testing, detailed below. The ELISA was used to test for the presence of *Plasmodium falciparum*, *P. vivax-210* and *P. vivax-247* circumsporozoite proteins to determine whether any of the female *Anopheles* mosquitoes were infected with malaria. These specific *Plasmodium* species were chosen because *P. falciparum* and *P. vivax* are the two most prevalent species in Madagascar (Barnadas, 2008).

The CDC provided all of materials (including antibodies) and training for the ELISAs. The *Plasmodium* Sporozoite ELISA protocol by Wirtz, Avery and Benedict was followed to perform the ELISA tests (Wirtz et al., 2011). In brief, the basic "sandwich" ELISA was used for detection of *Plasmodium* circumsporozoite proteins (Wirtz et al., 2011). The basic steps of this "sandwich" ELISA are: 1) coating the plate wells with anti-sporozoite monoclonal antibody, 2) adding mosquito homogenate to the wells, 3) adding

peroxidase-linked anti-sporozoite monoclonal antibody to the wells, 4) adding ABTS to the wells.

**PCR**

Before PCR was performed, the DNA was extracted from the abdomens of the collected female *Anopheles* mosquitoes. The Collins protocol for DNA extraction from a single mosquito, sourced from the CDC MR4 unit, was used for this process (Collins et al., 1987).

PCR was used to re-test the mosquitoes that were potentially positive for *Plasmodium* (*falciparum, P. vivax-210* or *P. vivax-247*) based on ELISA test results (i.e., mosquitoes that had results that exceeded the negative threshold). The Singh nested PCR assay was the specific protocol used to test for the presence of the *Plasmodium* genus in addition to a specific *Plasmodium* species (Singh et al., 1999). The primers and positive controls that were used in this study to determine species were specific for *P. vivax* and *P. falciparum*.

In addition, PCR is currently being used to determine the species of every female *Anopheles* mosquito. The ability of the *Anopheles* mosquito to become infected with the *Plasmodium* that infects humans depends on its species. The PCR protocol being used is a combined *An. gambiae* complex and ribosomal DNA type assay for Mopti/Savanna discrimination (Methods in anopheles, n.d.). In addition to detecting *An. gambiae* and Mopti/Savanna discrimination, this protocol is also capable of detecting *An. quadriannulatus*, *An. merus* and An. arabiensis (Methods in anopheles, n.d.).

Sequencing of the ITS2 region is currently being done for any mosquitoes that do not amplify with the *Anopheles* species tested for with the PCR protocol listed above.

### Data Analysis

Data analysis was performed using SAS 10.1 ® (SAS, Inc., Cary, North Carolina). General exploratory data analyses and bivariate relationships of variables of interest were examined. In addition, Poisson regression models were applied, while taking certain temporal factors (which are associated with the presence of mosquitoes) into account. The independent variables included in the model were: land-use, village, odor, moon illumination, precipitation, temperature (Table 3) and elevation (Table 1). The dependent variable was Anopheles. Additionally, the model was run to investigate the ratio of the number of *Anopheles* to the total number of mosquitoes by setting the total number of mosquitoes as the "offset" variable and Anopheles as the dependent variable in the model. The statistical analysis was used to determine the resulting effect of land-use on prevalence of *Anopheles* mosquitoes capable of carrying *Plasmodium*. This provided a level of risk in relation to presence of the vector, in certain land-use sites. Some data that were "missing at random" was left out of the analysis.

## Results

### Prevalence Data

A total of 2,033 mosquitoes, with 415 *Anopheles,* were trapped during the 21 nights of trapping. The average percent of *Anopheles* out of the total number of mosquitoes trapped each night in each village: 28.4% (SE=5.90) in Ambatolahy, 2.8% (SE=1.26) in Vohiparara, 38.6% (SE=13.20) in Ambodiaviavy, 53.7% (SE=12.45) in Menarano, 29.8% (SE=9.00) in Manokoakora, and 5.3% (SE=4.84) in Bevohazo (Table 2). And the average percent of *Anopheles* out of the total number of mosquitoes trapped

each night in each land-use site: 1.6% (SE=0.91) in forest sites, 31.4% (SE=6.74) in village sites, and 41.4% (SE=7.96) in agricultural sites (Figure 4, Figure 5).

### *Models*

A Poisson Regression model was performed to examine the relationship between the prevalence of *Anopheles* and independent variables in the study that may have affected their prevalence. The independent variables considered were: land-use (the independent variable we are investigating), odor, village, moon illumination, temperature, precipitation and proximity to animal pens. A full model was run with all seven independent variables. The following variables were significant: land-use (p-value: <0.0001), village (p-value: <0.0001), odor (p-value: <0.0001) and proximity to animals (p-value: <0.0001).

When the full model was performed to examine the relationship between the ratio of the number of *Anopheles* out of the total number of mosquitoes and all 7 of the independent variables listed above, the following variables were significant: land-use (p-value: <0.0001), village (p-value: <0.0001), proximity to animals (p-value: <0.0001) and moon illumination (p-value: 0.0047).

### *Mosquito Distribution Across Land-Use Sites*

The Poisson regression model with the number of *Anopheles* mosquitoes as the outcome, the Type 3 table tells us that land-use was a significant predictor (p-value = <.0001). Specifically looking at each land-use site separately, all categories of land-use sites (forest, village, agriculture) were significantly different from one another. In comparison to agricultural sites, the expected log counts of *Anopheles* for village sites

were 0.6328 lower and for forest sites the expected log count was 1.9513 lower than that of agricultural sites.

Additionally, the model was run to investigate the ratio of the number of *Anopheles* out of the total number of mosquitoes trapped in each site. In comparison to the agricultural sites, the log counts for the village sites were 0.63 less and for the forest sites the log counts were 3.38 less.

The same Poisson regression analysis was also used to investigate both the total mosquito numbers and the total insect numbers across land-use sites. Results showed that land-use was a significant predictor for these outcomes as well; however, the effect of land-use on mosquito and insect numbers was different than it was for *Anopheles* numbers. For total mosquito numbers, compared to agricultural sites, expected log counts for villages were 0.4131 times less and the expected log counts for forest sites was 0.7722 times greater. However, forest sites were expected to have the highest numbers of total mosquitoes, with forest having about 0.5266 times higher log counts than those of agricultural sites. For total insect counts, compared to agricultural sites, expected log counts for villages were 0.1895 times less and the expected log counts for forest sites was 0.1769 times greater.

### *Other Factors Significant to Anopheles Prevalence*

Since one trap at each land-use site was baited with an odor (3-Methyl-1-butanol) and the other was not. The success of these baits, in these trapping conditions, was important to analyze. Odor traps had higher trapping numbers in forest and agricultural sites, but not in village sites (Figure 7). Odor was not included in the final Poisson Regression model with *Anopheles* as the dependent variable, because the full model did

not show a statistical significance in the trapping numbers between the odor and non-odor baits (p-value=0.55). However, odor was a statistically significant independent variable in the Poisson final model with the ratio of *Anopheles* to total mosquitoes as the dependent variable. The SAS output indicates that the non-odor traps have a log count 0.666 higher than the odor traps, meaning that when examining the proportion of *Anopheles* out of total mosquitoes trapped, the non-odor traps had a higher percentage of *Anopheles* out of total mosquitoes.

When odor data were stratified by proximity to an animal pen, close proximity to animal pens appears to increase *Anopheles* numbers more than odor traps, even in agricultural sites. Although this study was not designed to look at animal influence on *Anopheles* prevalence, once traps were randomly placed within a land-use site, the proximity to animal pens was noted. Five traps were within 10 feet of an animal pen. These locations were: Ambodiaviavy agricultural trap with odor, Menarano agricultural trap without odor, Manokoakora village trap with odor, Manokoakora agricultural trap with odor and the Bevohazo village trap without odor. The relationship of *Anopheles* prevalence and proximity to an animal pen was investigated using Poisson regression analysis. Proximity to animals was a statistically significant independent variable in model looking at *Anopheles* trapping number as well as the model looking at *Anopheles* out of total mosquitoes trapping ratio. In the model with *Anopheles* as the dependent variable, traps that were not located in close proximity to animal pens had a log count 1.8122 lower (P-value-<0.0001) than that of the traps located in close proximity to animal pens. And in the model with the ratio of *Anopheles* out of total mosquitoes as the dependent variable, traps that were not located in close proximity to animal pens had a

log count 0.5142 lower (P-value-<0.0001) than that of the traps located in close proximity to animal pens.

## ELISA

ELISA results showed potentially positive results for *P. vivax-210* circumsporozoite proteins in three mosquitoes (Figure 6). These mosquitoes were trapped in three different locations: a Manokoakora agricultural site, a Manokoakora village site, and a Bevohazo village site.

## PCR Confirmation of Potential Positives

PCR conducted on the 3 potential positive samples from the ELISA analysis concluded that the samples were all negative for *Plasmodium*.

## ITS2

Preliminary data analyzing ITS2 sequences of *Anopheles* species suggest a potential lack of DNA in some of the mosquito samples. Without bands showing in the ITS2 analysis, there would not be reason to perform sequencing for these samples. The remainder of the female *Anopheles* mosquitoes will be tested with PCR to understand representative species for the captured mosquitoes.

# Discussion

*Review of Findings*

In this study, more insects and mosquitoes were trapped in forest sites, while more *Anopheles* were trapped in agricultural sites. *Anopheles* appear to be more prominent in areas of decreased concentration of other insects. A similar study looking at landscape factors influencing distribution of mosquito genera and frequency of virus infection concluded that risk for virus-positive mosquitoes was lowest in primary forests, higher in plantations and secondary forest, and highest in villages (Junglen et al., 2009). These data show a trend that increases in association with clearings and human presence (Junglen et al., 2009).

In addition, traps located in close proximity (< 3m) to animal pens were 2.6209 times more likely (p-value = <0.0001) to trap *Anopheles* mosquitoes than those traps that were not in close proximity to animal pens. These data may suggest a positive association between human malaria risk and proximity to livestock. However, the *Anopheles* trapped at locations in close proximity to livestock pens may be zoophilic species, in which case, a higher number of *Anopheles* captured may not pose a threat to humans tending these animals. Alternatively, if these *Anopheles* species are opportunistic feeders, then the large volume of livestock could be attracting more *Anopheles*, presenting equal risk to the livestock and the humans tending to them to be bitten.

Village was a statistically significant variable in both Poisson Regression models (*Anopheles* prevalence and *Anopheles* to total mosquito ratio) (p-value=<0.0001). However, temperature and precipitation were not statistically significant in either of the models because the values for these two independent variables were averages per day and

not per trap. Therefore, the village number represents temperature and precipitation. In addition, each village represents a certain population and density of people. All of these factors, specific to each village, could have an influential affect on the number of *Anopheles* trapped.

When examining the mosquito larva/pupa count data in each land-use site (Table 4), the majority of the sites had zero larva/pupa counted. In some cases it appeared as if this zero count data was accurately representative. For example, the villages of Ambodiaviavy, Menarano and Manokoakora had little to no sitting water and therefore little or no habitat available for the larva/pupa. In other cases like forests, presence of sitting water seemed likely in the site, but it had not been observed in the areas between the traps. Or in the case of rice paddies, all of the sitting water was too numerous to check, so there could have been larva in areas that went unchecked. In addition, it was raining during the majority of the time in Bevohazo (Table 3). In all other sites, larval dipping was not performed while it was raining because it is not effective since larva will not rest at the top of water pools during rain ("Larval Sampling," n.d.). Although, in Bevohazo the rain was unavoidable and therefore larval dipping was attempted in the rain, but nothing was found. Therefore, data for Bevohazo may not be representative of the mosquito breeding taking place in the village and surrounding land-use sites. However, due to the large amount of sitting water that was observed in and around the village, it seems very likely that mosquitoes were utilizing these water pools for breeding. Data indicate that Bevohazo had the highest average number of mosquitoes trapped per night (Table 2), thus supporting the argument that mosquito breeding was successful there even though larva was not found during the time of trapping.

### *Mosquito Breeding Sites: Importance of Larva Collection*

Investigation of mosquito breeding sites, by collection of larva, in each land-use site is one of the key aspects to understanding how land-use can affect malaria risk as well as other mosquito-borne diseases. Since trapping adult mosquitoes was the main element for data collection in this study, we were assuming that the different land-use sites are far enough apart from one another in order to conclude that the adult collection in a certain site is due to the habitat that land-use provides. However, if a distance small enough for a mosquito to travel in order to collect a blood meal separates land-use sites, then one land-use site could actually be influencing another land-use site in terms of the number of mosquitoes collected there. For example, in this study there were many cases where villages were surrounded by agricultural land and sometimes rice paddies ran right up next to a house at the edge of a village. If a large number of mosquitoes are trapped in the village, this may not be due to sitting water within the village, but it may be due to breeding sites within the agricultural land directly surrounding the village. Keeping this concept in mind, future studies should put a greater emphasis on larval collection in each land-use site in order to determine exactly what habitat is providing an improved environment for mosquito breeding and therefore what environment leadings to increased malaria risk.

### *Potential Positives for Plasmodium*

ELISA results showed potentially positive results for *P. vivax-210* circumsporozoite proteins in three mosquitoes (Figure 6). These mosquitoes were trapped in three different locations: a Manokoakora agricultural site, a Manokoakora village site, and a Bevohazo village site. However, as mentioned in Durnez et al., circumsporozoite

protein (CSP) ELISA has been found to greatly overestimate the entomological inoculation rate and falsely incriminate a vector when it may not actually be positive (Durnez et al, 2011). The false positive is most common for *P. falciparum* and for zoophilic species (Durnez et al, 2011). Although our three positive results were for *P. vivax-210*, and not *P. falciparum*, these vectors could be a zoophilic species. It is recommended that all CSP-ELISA positives be confirmed by either a second CSP-ELISA on the heated ELISA lysate or a *Plasmodium* specific PCR followed by PCR determination of *Plasmodium* species, if possible (Durnez et al., 2011). In this study, *Plasmodium* genus and species specific PCRs were used.

*Plasmodium* specific PCR showed no trapped mosquitoes being positive for *Plasmodium*. Further studies with more trapping numbers should be done in these same areas around Ranomafana National Park. Presumably, this would increase the percent chance of trapping a currently infected mosquito. Then the mosquito *Plasmodium* infection rates should be compared to human infection rates, distinguishing *Plasmodium* species.

### *Duffy Antigen*

Studies have shown that erythrocyte Duffy blood group negative people, mainly of African ancestry, are resistant to *Plasmodium vivax* infection (Menard et al., 2010). Thus, it has been thought that the Duffy antigen is necessary for *P. vivax* infection. In Madagascar's population of diverse ethnicities, there is a mixture of those that are Duffy blood group negative and those that are positive (Menard et al., 2010). In the Menard study, Duffy negative subjects in Madagascar that were infected with malaria, were tested to determine what *Plasmodium* species they were infected with. The results from this

study indicated *P. vivax* PCR positivity in both asymptomatic and symptomatic Duffy-negative people. Therefore, Menard et al. concluded that in Madagascar, *P. vivax* is no longer dependent on the Duffy antigen for establishing human blood-stage infection and disease (Menard et al., 2010). This makes malaria in Madagascar a unique example of parasite-host co-evolutionary adaptations to one another.

If future studies find contradictory results of mosquito *P. vivax* infectivity versus mainly *P. falciparum* infected people, this could be suggestive that in this area of Madagascar there are still Duffy-negative people that are resistant to *P. vivax* infection. This could be indicative of Duffy-negative people getting bitten by the *P. vivax* infected mosquitoes but not being infected by them. Although the Duffy-negative population is something to consider when this infective contradiction appears, it is also important to then question why the majority of infected mosquitoes are infected with *P. vivax* and not *P. falciparum*, if the majority of infected people have *P. falciparum*. The *Plasmodium vivax* infected mosquito population has to be maintained somehow. Since we know that the *P. vivax (*and *P. falciparum)* can only infect humans, there is not a possible animal reservoir to consider when trying to locate the source of this continued mosquito *Plasmodium* infection.

### *Dilution Effect*

Biodiversity plays an important role in vector-borne disease transmission (Schmidt, 2000). The "dilution effect" is the concept that when an environment is species rich (high biodiversity), there will be "incompetent reservoirs" that have a low capacity to infect feeding vectors and these will dilute the effect of the "highly competent reservoirs," which in turn reduces disease risk (Schmidt & Ostfeld, 2000). Deforestation causes

shrinking of habitat for many animal species and the potential for decreased biodiversity. Madagascar is considered a biodiversity hotspot due to its endemic species richness, but in the Malagasy villages in this study, deforestation could be leading to a decreased biodiversity. Therefore this leads to a higher likelihood that disease vectors are coming into contact with humans. Once a disease like malaria has infected a human, this parasite begins to thrive and allows another opportunity for mosquito infection, which could lead to more human infections.

### *Dilution Effect Applied to Livestock*

The concepts of the "dilution effect" can also be applied to livestock presence. As mentioned previously, trapping locations near animal pens appear to have increased numbers of *Anopheles*. The high density of $CO_2$ plumes from these animals could be attracting mosquitoes to the area, and in turn causing an increased risk of being bitten to humans that tend to these animals. However, the presence of livestock could also be increasing the number of "incompetent reservoirs," like increased biodiversity does in the dilution effect, and therefore actually decreasing the overall malaria transmission in the area. Since animals are not reservoirs for the common human malaria parasites, the livestock acts as a dead end host for the parasite. The increased prevalence of livestock in close proximity to humans could be decreasing the probability that the humans will be bitten. Every time that an animal is bitten instead of a human, the transmission of malaria is decreased.

Investigating the role of domesticated animals and human malaria infection is crucial. Observational data taken during this study indicated that in these particular populations animal ownership is common. But in addition to high levels of animal

ownership, what could be even more important is that these animals live in very close proximity to the people in these villages. Many times, poultry are kept inside of houses at night, pig pens are right next to homes, and zebu are constantly monitored in close proximity by their owner that fears the possibility of theft. Based on the observed relationship between animal presence and increased mosquito populations, the association between animal ownership and malaria infection should be further investigated in this area of Madagascar.

### *Zooprophylaxis*

More specifically, the concept of zooprophylaxis should be studied in Madagascar (Saul, 2003). The term zooprophylaxis is the concept of animals attracting blood-feeding insects in order to divert them from humans (Saul, 2003). By using animals that are dead end hosts of diseases, meaning that they cannot act as a reservoir for the disease and therefore the disease cannot further spread from these animals, transmission rates are reduced. Therefore anytime a malaria-infected mosquito bites a non-human animal, this reduces the risk of a human being infected with malaria in the surrounding area.

The effectiveness of zooprophylaxis depends on the feeding preferences of the mosquitoes. The lower the proportion of the blood meals that a mosquito population takes from a human (the human blood index, HBI), the more useful zooprophylaxis can potentially be (Saul, 2003). But if HBI is too low, zooprophylaxis may not be useful. In fact, studies have collected data showing that zooprophylaxis is actually associated with higher prevalence of disease in some cases (Bouma & Rowland, 1995).

*Modeling Zooprophylaxis to Estimate Benefit*

The proposed effectiveness of zooprophylaxis for malaria control in specific settings can be modeled. Sota and Mogi developed a model of a mosquito population that has two blood meal hosts, one of man and the other of domestic animals, in order to study the impact of domestic animals on the frequency that a mosquito bites a human and the resulting impact on the endemicity of human malaria (Sota & Mogi, 1989). The concluding analysis of the model suggests that when domestic animals are present and easily fed upon by the mosquitoes, mosquito density increases due to increased availability for successful blood-feeding, and therefore increases the potential for mosquitoes biting a human (Sota & Mogi, 1989). Malaria transmission was only modeled to decrease when there were an extremely large number of easily accessible domestic animals, in comparison to humans (Sota & Mogi, 1989). This model is important to consider when discussing the potential use of zooprophylaxis for malaria control.

Although the Malagasy villages in this study have livestock living in close proximity to the people, the proportion of livestock to humans may not be high enough to aid in malaria control. In fact, it may have the potential to have the opposite impact. A potential study to investigate the effectiveness of zooprophylaxis, and the accuracy of this zooprophylaxis model to estimate benefit, could involve trapping mosquitoes in villages with varying ratios of human to livestock population. Specific traps would need to be selected in order to increase the likelihood of trapping blood-fed mosquitoes. For example, resting boxes were best to promote increased chances of trapping blood-fed *Culicine* mosquitoes in a study in Riverside County, CA (Sandhu, 2013). By trapping blood-fed mosquitoes, the blood meal could be analyzed with PCR in order to determine

whether it is from a human or an animal. The ratio of human to animal population in each village could then be compared to the ratio of mosquitoes with human blood meals to those with animal blood meals. The assumption is that there would be some type of threshold when animal population is high enough, in comparison to the human population that the mosquito blood meals would then be disproportionally high from animals.

If certain villages do not contain a high enough population of animals, in comparison to human population, to benefit from zooprophylaxis then other control measures would need to be put into place. One such approach has been to also use insecticide spraying in villages where animal population is too low to break the necessary threshold for zooprophylaxis benefits on its own.

### *Odor vs. Non-Odor Baited Traps*

Another factor in trapping success was the odor (3-Methyl-1-butanol) bait (Mukabana, 2012). Since one trap at each land-use site was baited with this odor and the other one was not, the comparison on trapping numbers of each trap is important to analyze. Although odor was not statistically significant in the Poisson Regression model with Anopheles as the dependent variable, it was for the model with the ratio of *Anopheles* to total mosquitoes as the dependent variable. The SAS output indicates that the non-odor traps have a log count 0.666 higher than the odor traps, meaning that when examining the proportion of *Anopheles* out of total mosquitoes trapped, in general the non-odor traps had a higher percentage of *Anopheles* out of total mosquitoes. Based on mosquito and *Anopheles* trapping numbers in each land-use site, stratified by odor/non-odor baited trap, our results show higher trapping numbers for odor traps in forest and agricultural sites, but not those in village sites (Figure 7). These data indicate that the

non-odor baited traps are better in the villages for obtaining higher ratios of *Anopheles* to mosquitoes. However, this trend could just be by chance, since the power of this study is low due to the limited number of trapping nights and trapping locations that the time allowed for.

One explanation for the lack of mosquito and *Anopheles* preference for odor baited traps in villages could be that mosquitoes, and *Anopheles* mosquitoes in particular, are more attracted to the humans in the village than they are to the odor. Therefore, possibly both the odor and non-odor baited traps had the same level of attractant to the mosquitoes in the villages, both secondary to the smell of humans. Odor baited traps have been proposed as a way to divert mosquitoes from humans in villages (Okumu, 2010). Although these findings do not oppose the use of odor-baited traps as tools for malaria control, they suggest that higher doses of certain odor-baits may need to be used when in village environments, due to the high level of attractant that humans naturally produce (Okumu, 2010). Or possibly, testing of this odor has successfully increased the attraction of *Anopheles* mosquitoes in other countries, but human odors and/or mosquito odor preference could vary globally or even regionally. Perhaps mosquitoes in this region of Madagascar prefer human odor to this 3-Methyl-1-butanol odor bait.

### *Environmental Risk Factors of Malaria Transmission*

There are many malaria transmission factors to consider when developing malaria interventions, solutions, treatments and possibly future vaccines. These factors include: mosquito life span, lifecycle of mosquito and parasite, human to mosquito interaction and their feeding preference/characteristics. When examining ways of preventing malaria infection in people, without a current vaccine available for use, the existing individual

prevention methods include prophylaxis, insect repellant, bed nets, screens, etc. However, some of these prevention methods are not always practical, such as insect repellant use everyday, and some of them are not accessible or not used properly, such as bed nets. Therefore, it is important to step back and address some of the larger-scale environmental risk factors for malaria transmission. These risks include mosquito breeding sites and land-use change.

Certain agricultural practices, such as irrigation, may alter the nutrient content of soil, as well as watershed dynamics, in ways that increase breeding of mosquitoes (Vittor, 2009). For example, irrigation can cause an increase in the number of mosquitoes, which sometimes leads to an increase in malaria prevalence (Mutero et al., 2006). Mutero, McCartney and Boelee present two examples to support this claim: in Burundi malaria parasite prevalence in irrigated rice fields was about 24-69% versus about 5-30% in non-irrigated cotton-growing fields; and in Kenya, the Hola cotton and vegetable irrigation was reported to have a malaria prevalence about 54% higher than that in nearby non-irrigated areas.

Although both of these agricultural cases show an increase in malaria parasite prevalence, an increase in mosquito population does not always mean an increase in malaria (Mutero et al., 2006). Malaria prevalence also depends on factors such as prevention measures that the local people are taking (such as using insecticide-treated nets and seeking treatment) and whether or not the increasing mosquito population is mainly a species that prefers to feed on humans or not (Mutero et al., 2006).

### *Species Determination in this Study*

The primers and positive controls that were used to test for the presence of the *Plasmodium* parasite were specific for *P. vivax* and *P. falciparum*. *P. vivax* was chosen in order to investigate the potential positive results from the previous ELISA. And testing for *P. falciparum* was chosen due to its high prevalence in this region of Madagascar and the slightest possibility that a mosquito infected with *P. vivax* could also be infected with *P. falciparum*, but the ELISA only detected the *P. vivax*.

In addition, PCR is being used to determine the species of every female *Anopheles* mosquito. The ability of the *Anopheles* mosquito to become infected with the *Plasmodium* that infects humans depends on its species. The published distribution of *Anopheles* species in Madagascar shows that *Anopheles gambiae* would be dominant in the region where our traps were set (Pock Tsy, 2003); however, in other humid bioclimates in Madagascar, *Anopheles arabiensis* have been found (Pock Tsy, 2003). The WHO 2013 World Malaria Report for Madagascar also describes species of *Anopheles* in the region as being *An. gambiae* and *An. arabiensis,* but also includes *An. funestus* ("World malaria report," 2012). Therefore, the PCR protocol being used is a combined *An. gambiae* complex and ribosomal DNA type assay for Mopti/Savanna discrimination (Methods in anopheles, n.d.). In addition to detecting *An. gambiae* and Mopti/Savanna discrimination, this protocol is also capable of detecting *An. quadriannulatus*, *An. merus* and An. arabiensis (Methods in anopheles, n.d.). Sequencing of the ITS2 region is currently being done for any mosquitoes that do not amplify with the *Anopheles* species tested for with the PCR protocol listed above.

*Importance of Species Determination of Anopheles and Plasmodium*

Determining the species of the *Anopheles* mosquitoes is also important in order to determine their susceptibility to *Plasmodium* infection. Although a certain land-use may increase mosquito breeding, not only is it important to determine what genus the mosquito is but it is also important to determine what the species is and possibly even the particular strain (Jaramillo-Gutierrez et al., 2009). For example, *An. gambiae* (G3 strain) is highly compatible with *P. falciparum* (3D7 strain) parasites (Jaramillo-Gutierrez et al., 2009).

A*nopheles* species is important to know in order to determine feeding habits, preferred breeding sites, survival rates, and ability to support malaria parasite development (Beier, 1998). In a literature review conducted by Yasuoka & Levins, analysis of data indicated deforestation and agricultural development caused varying changes in the density of different *Anopheline* species (Yasuoka & Levins, 2007). For example, when looking at three different *Anopheline* species of interest in Madagascar: *An. arabiensis, An. gambiae* and *An. funestus*, all three have been found to increase or decrease in density due to varying types of land-use. *An. arabiensis* appears to increase density due to irrigation development (Yasuoka & Levins, 2007). *An. gambiae* has been found to increase density due to both irrigation development and rice development (Yasuoka & Levins, 2007). *An. funestus* has also been found to increase density due to rice development, but also to decrease density due to deforestation (Yasuoka & Levins, 2007). In the deforested areas of Madagascar, based on these data, irrigated agricultural sites would have the highest potential for *Anopheles* density increase. The Yakuoka and Levins study supports the underlying difficulty of linking malaria epidemiology to deforestation and agricultural development, due to its extreme complexity (Yasuoka &

Levins, 2007). In addition, knowing what type of *Plasmodium* species are present in a certain area is important in order to be aware of signs and symptoms that are unique to each species of the parasite.

Therefore, by determining what locations are prone to be ideal breeding sites for mosquitoes, we can target environmental interventions for disease management in addition to targeting malaria prevention and treatment initiatives. Knowledge of vector location is the first step to detailing an efficient plan for malaria control that helps reduce the greatest amount of disease with the funds available.

### *Limitations to the Study*

A major limitation to this study was the lack of time and trapping materials that limited the number of trapping locations and trapping nights that were completed. Therefore, this decreased the statistical power of the study.

Trapping only during the dry season poses another limitation to this study. While there are benefits to trapping in the dry season, such as identifying malaria "hot spots" so that prevention methods/ prophylaxis distribution can be targeted, it is also less likely that an infected mosquito will be trapped at this time. The probability of trapping a currently infected/infective mosquito is low due to the many factors involved. For example, a study done in Tanzanian villages, looked at malaria infection potential of *Anopheline* mosquitoes sampled by 160 light traps each trapping night, for June, July and August, which is during the dry season in Tanzania and Madagascar. The sporozoite rate for *An. gambiae s.l.* was 0.069, 0.25 and 0.09 respectively. And for *An. funestus* the sporozoite rate was 0.035 in June, 0.108 in July and 0.07 in August (Shiff, 1995). Also, even though malaria has developed to very high transmission rates, the majority of mosquitoes that

acquire gametocytes during blood feeding on a malaria infected host, do not actually support the development of the parasite into the sporozoite stage (Beier, 1998).

In addition, the diversity of the land in Madagascar makes it hard to compare malaria transmission findings in one region to another. For example, this study took place in Ranomafana National Park, which is a continuous humid tropical forest with perennial malaria transmission (Wright, 1997; PMI, n.d.). The results from this study cannot be directly applied to malaria control programs in the central highland region of Madagascar where there is unstable seasonal malaria transmission (PMI, n.d.).

### *Future Directions*

Based on previous studies and models, Pielke et al. concluded that evidence supports that short-term biophysical and long-term biogeochemical changes significantly influence weather and climate (Pielke, 1998). A study by Lindblade et al. investigated recent outbreaks of malaria in southwestern highlands of Uganda, where malaria transmission rarely occurs (Lindblade, 2000). Temperature can directly impact malaria transmission. When temperatures are near the lower limit for survival and development of the parasite and the mosquito, small fluctuations in temperatures could greatly impact the density of the parasite and mosquito in a certain area (Lindblade, 2000). In the Lindblade et al study, data indicated the number of *An. gambiae s.l.* per house increased by 77% for every 1 degree Celsius increase in average minimum temperature (Lindblade, 2000). Discussion of this finding suggested that local temperature changes were a major factor in the increase of malaria transmission and not just due to larval habitats caused by swamp cultivation, like many other studies have hypothesized (Lindblade, 2000). Therefore, more advanced studies are needed that record the temperature of different

land-use sites, as deforestation is taking place and agricultural sites are being developed, and simultaneously take mosquito prevalence data each month.

For *P. falciparum*, the lower temperature limit for parasite survival is about 16 degrees Celsius (Lindablade, 2000). Slight temperature changes could determine whether or not the *Plasmodium* parasite can survive in a certain area. In addition, the extrinsic incubation period (EIP) of *P. falciparum* can be reduced by days when temperature is increased by less than one degree Celsius (Lindblade, 2000).

## Conclusion

Data from this study support my initial hypothesis that agricultural sites would have a higher prevalence of *Anopheles* mosquitoes than forest or village sites. However, it is unclear whether or not agricultural sites pose a higher risk for malaria. Many factors such as temperature, proximity to villages and *Anopheles/Plasmodium* species prove to play important roles in malaria risk. Although sample size precluded detailed examination of this relationship, proximity to domesticated animal pens was associated with higher prevalence of *Anopheles*, thus emphasizing the importance of yet another factor critical in analyzing malaria risk. Therefore, further investigation will be required to develop specific predictions of risks for malaria transmission and potential for malaria interventions.

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# Appendix: Tables and Figures

**Table 1. Location Coordinates and Dates of Sampling for Mosquito Trap Sites in Six Villages near Ranomafana National Park, Madagascar.**

| | Trap | Location | Elevation (m) |
|---|---|---|---|
| **Ambatolahy (6/17/13-6/21/13)** | Forest | S 21°15'05.6" E 047°25'21.6" | 1011 |
| | Forest w/odor | S 21°15'07.5" E 047°25'23.3" | 930 |
| | Village | S 21°14'57.3" E 047°25'48.2" | 865 |
| | Village w/odor | S 21°14'58.8" E 047°25'47.3" | 856 |
| | Agriculture | S 21°15'00.6" E 047°25'48.1" | 872 |
| | Agriculture w/odor | S 21°15'02.1" E 047°25'47.7" | 876 |
| | | | |
| **Vohiparara (6/30/13-7/4/13)** | Forest | S 21°14'17.8" E 047°23'41.4" | 1129 |
| | Forest w/odor | S 21°14'08.5" E 047°23'46.9" | 1129 |
| | Village | S 21°14'20.7" E 047°22'53.0" | 1133 |
| | Village w/odor | S 21°14'20.1" E 047°22'54.7" | 1136 |
| | Agriculture | S 21°14'11.4" E 047°23'07.0" | 1129 |
| | Agriculture w/odor | S 21°14'22.5" E 047°22'58.2" | 1131 |
| | | | |
| **Ambodiaviavy (7/7/13-7/10/13)** | Forest | S 21°15'26.4" E 047°28'34.1" +/- 10 m | 744 |
| | Forest w/odor | S 21°15'23.4" E 047°28'34.1" +/- 10 m | 781 |
| | Village | S 21°15'48.8" E 047°29'06.0" | 640 |
| | Village w/odor | S 21°15'50.8" E 047°29'05.7" | 642 |
| | Agriculture | S 21°15'45.6" E 047°29'03.3" | 623 |
| | Agriculture w/odor | S 21°15'50.4" E 047°29'09.2" | 619 |
| | | | |
| **Menarano (7/20/13-7/24/13)** | Forest | S 21°17'27.9" E 047°27'16.3" | 834 |
| | Forest w/odor | S 21°17'27.2" E 047°27'20.2" | 812 |
| | Village | S 21°17'26.3" E 047°28'07.9" | 716 |
| | Village w/odor | S 21°17'25.3" E 047°28'07.2" | 715 |
| | Agriculture | S 21°17'34.9" E 047°28'06.5" | 686 |
| | Agriculture w/odor | S 21°17'32.2" E 047°27'59.6" | 688 |
| | | | |
| **Manokoakora (7/29/13-8/1/13)** | Forest | S 21°17'10.7" E 047°32'46.1" | 644 |
| | Forest w/odor | S 21°17'11.0" E 047°32'47.1" | 646 |
| | Village | S 21°17'12.0" E 047°32'36.1" | 612 |
| | Village w/odor | S 21°17'12.5" E 047°32'40.2" | 612 |
| | Agriculture | S 21°17'17.7" E 047°32'46.0" | 605 |
| | Agriculture w/odor | S 21°17'15.0" E 047°32'42.5" | 616 |
| | | | |
| **Bevohazo (8/3/13-8/6/13)** | Forest | S 21°12'20.4" E 047°30'10.0" | 720 |
| | Forest w/odor | S 21°12'21.8" E 047°30'07.0" | 689 |
| | Village | S 21°12'37.0" E 047°29'54.5" | 616 |
| | Village w/odor | S 21°12'30.6" E 047°29'54.7" | 616 |
| | Agriculture | S 21°12'37.9" E 047°29'52.0" | 616 |
| | Agriculture w/odor | S 21°12'36.1" E 047°29'54.4" | 602 |

**Table 2. Summary and comparison of *Anopheles* and total mosquitoes trapped in each village per night in Six Villages near Ranomafana National Park, Madagascar.**

| Village | # Trap Nights | Average # *Anopheles* Trapped per Night (SE) | Average # Mosquitoes Trapped per Night (SE) | Average % *Anopheles* among total Mosquitoes Trapped per Night (SE) |
|---|---|---|---|---|
| Ambatolahy | 4 | 7.8 (0.73) | 27.3 (1.42) | 28.4 (5.90) |
| Vohiparara | 4 | 1.8 (0.23) | 63.5 (5.02) | 2.8 (1.26) |
| Ambodiaviavy | 3 | 24.3 (6.21) | 63.0 (9.04) | 38.6 (13.20) |
| Menarano | 4 | 25.5 (4.35) | 47.5 (5.96) | 53.7 (12.45) |
| Manokoakora | 3 | 57.7 (5.39) | 193.3 (12.63) | 29.8 (9.00) |
| Bevohazo | 3 | 13.3 (2.20) | 253.3 (23.66) | 5.3 (4.84) |

Table 3. Environmental factors of each mosquito trapping period in six villages near Ranomafana National Park, Madagascar. (TEAM, 2013)

| Dates | Village | Range of Temperature (Celsius) | Average Temperature (Celsius) (SE) | Average Temperature During Trapping (6pm-6am) (Celsius) (SE) | Total Precipitation (mm) | Lunar Phases |
|---|---|---|---|---|---|---|
| 6/17/13--6/21/13 | Ambatolahy | 6.99 - 17.04 | 12.57 (0.05) | 11.13 (0.07) | 43.69 | waxing 58.3% - waxing 91.7% |
| 6/30/13--7/4/13 | Vohiparara | 8.47-20.71 | 14.21 (0.08) | 13.2 (0.08) | 12.19 | waning 50%-waning 25% |
| 7/7/13--7/10/13 | Ambodiaviavy | 6.95-17.01 | 12.54 (0.06) | 11.39 (0.06) | 1.78 | waning 8.3%-waxing 8.3% |
| 7/20/13--7/24/13 | Menarano | 5.08-17.95 | 12.19 (0.07) | 10.56 (0.09) | 10.16 | waxing 91.7%-full moon |
| 7/29/13--8/1/13 | Manokoakora | 10.35-20.84 | 13.47 (0.08) | 12.6 (0.06) | 41.4 | waning 50%-waning 33.3% |
| 8/3/13--8/6/13 | Bevohazo | 9.73-14.31 | 11.6 (0.02) | 11.26 (0.03) | 120.65 | waning 25%-waning 8.3% |

**Table 4. Mosquito larva/pupa collection totals in each land-use trapping site (not specific to each trap), in six villages near Ranomafana National Park, Madagascar.**

| | Forest | Village | Agriculture |
|---|---|---|---|
| **Ambatolahy** | 0 | 0 | 0 |
| **Vohiparara** | 11 | 5 | 10 |
| **Ambodiaviavy** | 0 | 0 | 0 |
| **Menarano** | 0 | 0 | 6 |
| **Manokoakora** | 0 | 0 | 27 |
| **Bevohazo*** | 0 | 0 | 0 |

*Note: It was raining during every larval dip

**A.**

**B.**

**C.**

**Figure 1. Examples of trapping sites in and around six villages near Ranomafana National Park, Madagascar. A. Forest-trapping site. B. Village-trapping site. C. Agricultural-trapping site.**


**Figure 2. Example of CDC miniature light trap setup, in six villages near Ranomafana National Park, Madagascar**

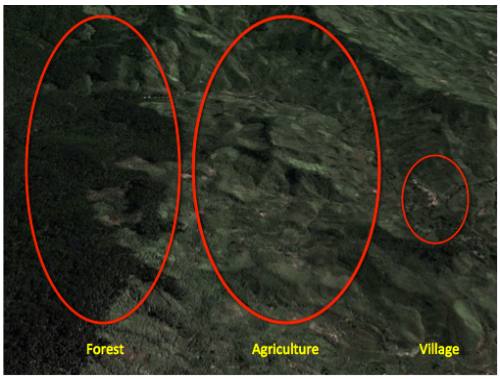


**Figure 3. Google Earth image of the village of Menarano and surrounding agricultural and forest sites near Ranomafana National Park, Madagascar.**

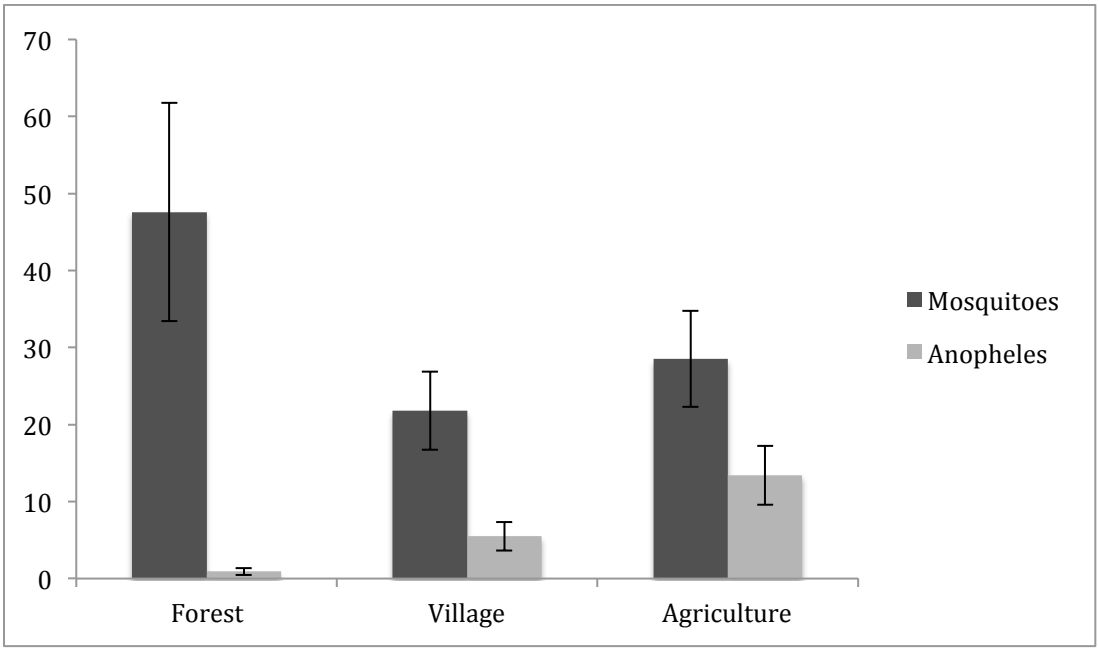


**Figure 4. Average Number of Mosquitoes and *Anopheles* Trapped Each Night by Land-Use Type, with standard error bars, in Six Villages near Ranomafana National Park, Madagascar.**

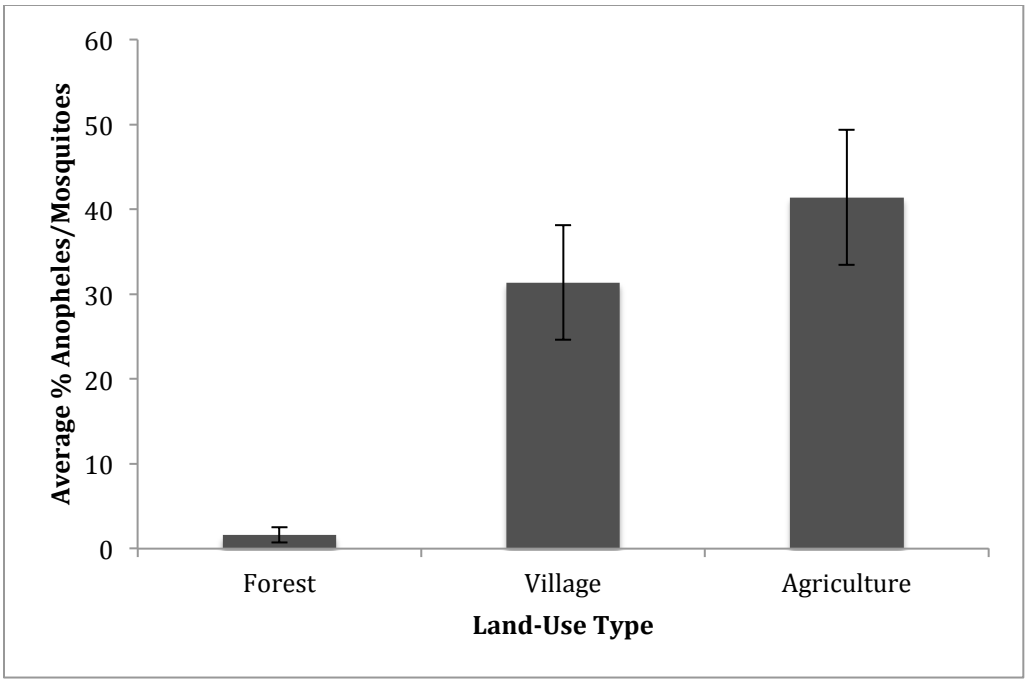


**Figure 5. Average Percent of *Anopheles* of Total Mosquitoes Trapped Each Night by Land-Use Type, with standard error bars, in Six Villages near Ranomafana National Park, Madagascar.**

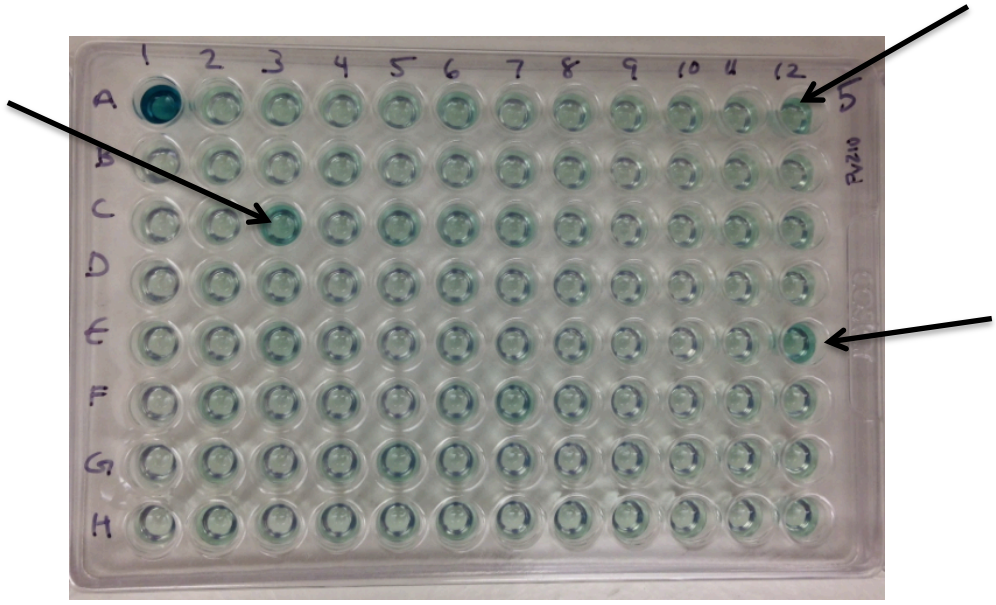


**Figure 6. ELISA plate that includes potential positive (for *pv-210*) mosquito samples trapped in Six Villages near Ranomafana National Park, Madagascar.Wells A12, C3 and E12 hold the potential positives.**

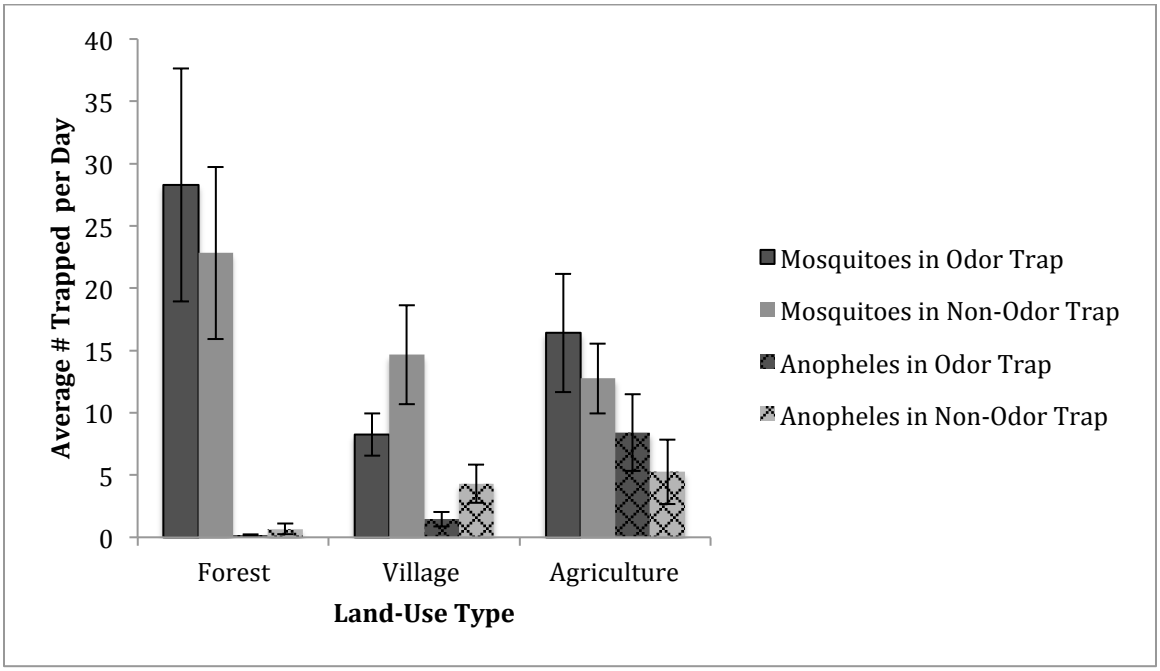


**Figure 7. Average number of total mosquitoes/*Anopheles* trapped per night in each land-use type Six Villages near Ranomafana National Park, Madagascar, with standard error bars, stratified by odor/non-odor trap**