Prevalence of *Plasmodium* Spp. in Northern Michigan

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Abstract:

Prevalences of passerine malaria were compared at the maple river MAPS site this year to that recorded two years ago in the same site. To identify this apicomplexan, birds were caught in mist nets and blood smears were taken from them, which were then stained and viewed under a microscope in 100x oil immersion. Of 44 passerines, two birds infected with *Plasmodium* sp. were found. These data were compared to the seventeen infected birds in 159 caught from the study in 2006. A Chi-squared test revealed that there was no significant change in the malaria prevalence in passerines at the Maple River site and furthermore that a larger sample size and better malaria detection methods ought to be used in the future.

Malaria is known worldwide as a great killer of humans, but the malaria that was researched for this study is in birds. The lifecycle of malaria begins in the female mosquito gut as an oocyst, which then bursts and yields many sporozoites, the infective stage for birds. The sporozoites are transmitted to the bird when the mosquito goes to take a blood meal from the bird. Once inside the bird, the sporozoites migrate to the liver, where they multiply as merozoites until they burst out of the liver cell and go into the blood stream to infect red blood cells. The merozoites again replicate in the red blood cells, and then burst all at the same time; in humans this causes intense pain and fever. The merozoites then infect more blood cells, but some of them become gametocytes; the female macrogametocyte or the male microgametocytes. These cells later get sucked up by another mosquito, and end up in the mosquito gut. When the gametocytes get into the mosquito gut, they meet up and a microgametocyte fertilizes a macrogametocyte to form a zygote in a mobile cell called an ookinete. This ookinete moves to the mosquito gut wall and forms a cyst there called an oocyst, which begins the process again (Valkiunas 2004). Avian malaria is very identical to human malaria in its lifecycle, but has one very important difference that allows it to exist in places where human malaria has long since been eradicated, which is that it can use many different genera of mosquitoes as a host, as opposed to human malaria, which can only use mosquitoes of the genus *Anopheles*. Because mosquitoes of the genus *Anopheles* have been eradicated in North America, but other genera have not, avian malaria is able to persist where human malaria could not. In the continental U.S., avian malaria tends to be not very pathogenic, likely because the birds coevolved with the disease, but in naïve hosts, such as native Hawaiian birds, it can be very pathogenic and a conservation nightmare (Beadell *et al.* 2006).

Although Mixson *et al.* (2006) examined all haemosporidians that might be in the blood of birds in the UMBS area of northern Michigan, but focused mainly on what will be described in this study as malaria, which is *Plasmodium* spp., rather than the broad definition that was used by Mixson *et al.* (2006), which included Haemoproteus and Leukocytozoan. The present study is a follow-up to the findings of study in 2006 regarding malaria in passerines in the mist-netting site at the Maple River. The reason that passerines were the subject of this study was because the previous study had looked only at passerines and passerines are the most common order of bird that is found at the site. The goal of the follow-up was to determine whether the malaria prevalence in passerines at the Maple River mist-netting site had changed in the two years between studies. The hypothesis tested was that the prevalence had not changed between the two years, which could have been disproven by finding significant difference in a Chi-squared test. This hypothesis was used because there is no reason that is known for why malaria prevalence would change. At the same time, the data that were gathered were used to make a confidence interval within which there is a 95% chance that the true prevalence of malaria in passerines at the site exists. The dependent variable that was tested was time, specifically the two years between the original study and this one, and the independent variable was the prevalence of malaria. To satisfy these purposes, a large number of birds needed to be caught, ideally as many as were recorded in the original study, which was 159 (Mixson *et al.* 2006). To accomplish this, mist-netting procedures were used and the birds were then bled to make blood smears, which were analyzed with a microscope for five minutes to determine if the bird was infected. While more sophisticated studies use PCR to detect malaria (Feldman *et al.* 1995), this study did not have access to modern PCR techniques and instead relied on Giemsa-Wright staining and light microscopy.

Materials and Methods:

Each day, banding began at approximately 6:30 am. Bird sampling was done at the north Maple River MAPS site that had been set up previously. Twelve different sites were used within the area, with between four and ten being active at any given time. Mist-netting sites varied in the distance from Maple River. Birds were caught in mist-nets, which are black nylon mesh nets that extend about ten feet off the ground and about 35 feet in length. The premise of the nets is that birds will fly into them, and then fall into the pockets that are along the sides. After they were caught, the birds were taken back to the central location so that measurements could be taken. The wing chord length of each bird was measured using a specialized ruler and body mass was measured using a tiny scale. The age and sex were determined, if possible by various methods. For this study, those were the only characteristics that were measured, but multiple studies were collaborating and more data were taken. At the same time, the birds were sized for a band, and then banded, which involves clamping a numbered piece of metal down on the leg of the bird such that it is able to slide, but cannot come off, using a specialized pair of pliers. After all the data had been recorded for a given bird, blood was taken from the brachial artery using a sterilized needle and pipette, after carefully removing the coverts and sterilizing the area with rubbing alcohol. Two smears were made of each bird so that there was a backup in case one of the slides did not turn out well. After the blood smears were made and had dried, they were fixed in methanol within an hour and later stained using Giemsa-Wright stain. After the slides were dry for the final time, they were stored in a slide box to prevent anything from damaging them. Only the primary slide was stained in most cases, unless the primary slide was in some way damaged, in which case the secondary slide was then stained and used. This procedure was done for each bird that was caught, with the exception of very young birds, which likely would

be harmed by the process, and birds that had previously been sampled. Birds were caught until 10:30 am, at which point the nets were closed and removed.

To detect malaria on the slides, the area of interest was first located under the 45x (high-dry) lens of the microscope, and then the lens was moved halfway between that and the 100x oil lens, and a drop of oil was placed on the slide. The slides were observed under oil immersion for five minutes. During that time, mostly the leading edge was examined, and while all abnormal things were noted, it was a trophozoite on a gametocyte within the red blood cell, with black dots, that was indicative of malaria. When malaria was discovered, it was noted for later verification. After looking at a slide under oil immersion, to remove the oil, chloroform was added on top of lens paper, on top of the oil and then the lens paper was slowly withdrawn parallel to the slide, so that the oil was removed without taking any of the cells with it. This process was repeated for every slide that was stained.

Results:

From our study, forty six birds were caught and sampled, and among those, forty four were passerines. The two woodpeckers that were caught were disregarded in survey, and both had no malaria.

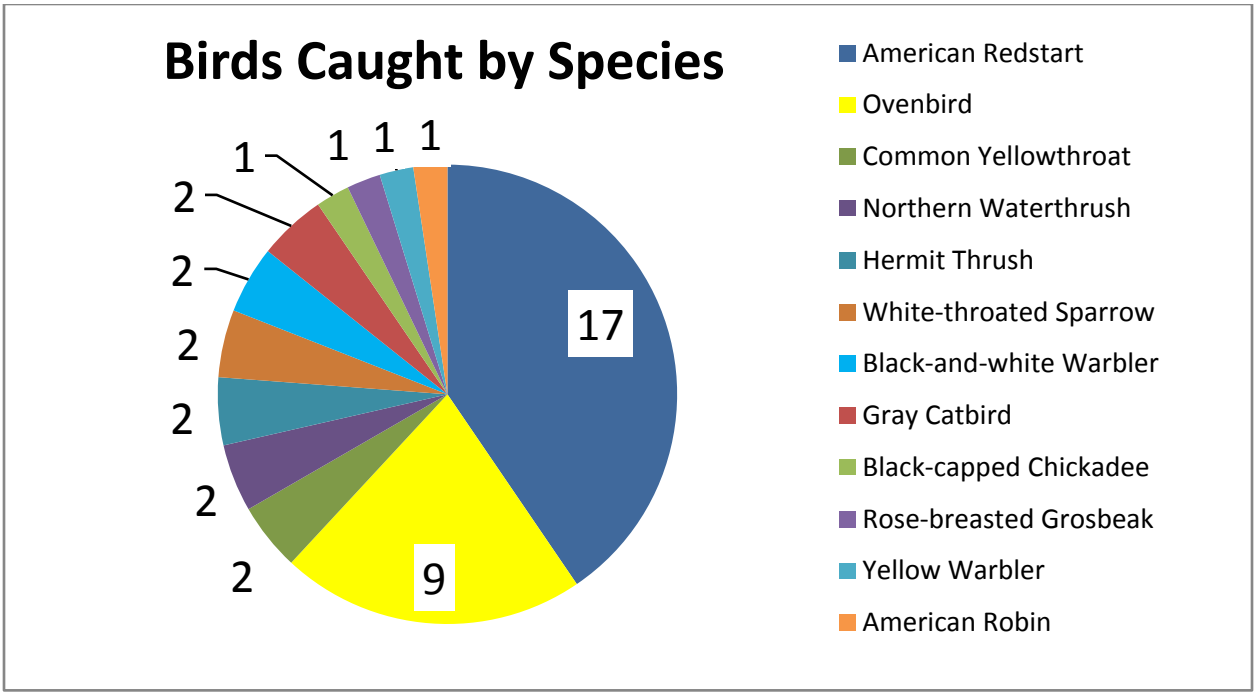

Fig. 1 Summary of species sampled, a numerical pie chart showing how many of each bird was caught, and what their proportion is to the total number of birds.

Two of the 44 passerines (4.55% prevalence) that were caught were found to be positive for *Plasmodium* sp.. These two birds were a White-throated Sparrow and an Ovenbird. To test the null hypothesis that the malaria prevalence in passerines at the Maple River banding site had not changed over the two years between the original and follow-up study, a Chi-squared test was performed using the data from this study and the original study, which found seventeen infected out of one hundred fifty nine caught, for a percentage of 10.69%. Using these two data sets, a

chi-squared test was done, with the following calculation: $\chi^2 = \Sigma \frac{(E-O)^2}{E}$ where E=expected and O=observed. Each part was done on its own, the expected and observed values for the original infected, original uninfected, follow-up infected and follow-up uninfected and then plugged into the equation to yield .35, .03, 1.09 and .11 respectively. These values were then added together, which gave a value of 1.58 which, when looked up on a chi-squared chart, is greater than 3.84, which gives a p-value that is greater than .05, which makes the data not statistically significant, and fails to reject the null hypothesis, which means that the data have not been proven to be different.

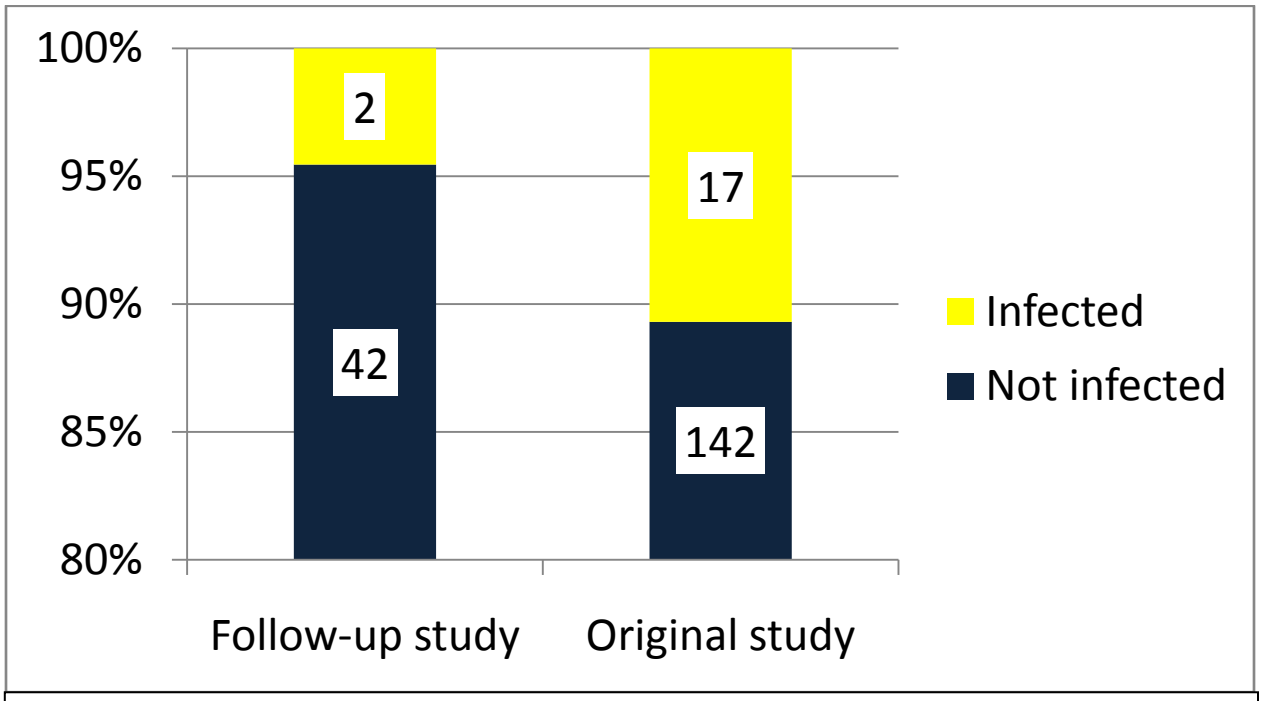

Fig. 2 A comparison of the prevalence of malaria in the passerine birds caught at the Maple River mist-netting site between the original study and the current study. The bars are not to scale.

A confidence interval was calculated from the data that was obtained, which gave, with 95% confidence, the interval in which the true value of malaria prevalence in passerines at the Maple

river site exists. The confidence interval calculation was done using the equation $\hat{p} \pm z^* \sqrt{\frac{\hat{p}(1-\hat{p})}{n}}$, where $\hat{p}$ is the sample proportion, $z^*$is a value from a table, for 95% confidence intervals, it is 1.96, and n is the sample size that the sample proportion was created from. In this case, $\hat{p}$ = 19/203, or .0936, and n = 203, the total number of passerines sampled between the studies. That made the equation $.0936 \pm 1.96\sqrt{\frac{.0936(1-.0936)}{203}}$ = .0936 ± .0400, which gives a confidence interval of (.0536, .1336). This means that there is a 95% chance that the actual prevalence of malaria in passerines at the Maple River site is between 5.36% and 13.36%.

When the 27% detection rate of malaria using microscopy techniques found by Jarvi *et al.* (2002) was factored into the equation, a more accurate confidence interval was created, by multiplying .0936 by 1/.27 to get .3467, and using that as the $\hat{p}$. This made the equation $.34665 \pm 1.96\sqrt{\frac{.3467(1-.3467)}{203}}$, which gave a confidence interval of (.2812, .4121). This meant that the corrected actual prevalence of malaria in passerines at the Maple River site is between 28.12% and 41.21%, assuming that only 27% of infected birds were detected through microscopy.

The results, specifically from the chi-squared test, do not disprove our hypothesis, the null hypothesis, that there has been no change in the malaria prevalence in the passerines at the Maple River site between 2006 and 2008.

Discussion:

Using both of the data sets together, a confidence interval of 95% was made for the malaria prevalence there, which is between 5.36% and 13.36%. The first thing to mention about the data is that the fact that some of the data that were gathered fell outside of the confidence interval that was generated using that data does not mean that the data are statistically significant, or that the malaria prevalence is necessarily changing, just that the data gathered was on the low end of what could be expected, and alone would have generated a much lower confidence interval.

The chi-squared test was not done under the best circumstances, as one of the expected values was less than 5, in which case the chi-squared test is not supposed to be valid. Lacking further samples to improve our expected value, the decision was made to perform the test despite this fact. It certainly would seem possible that if additional samples were gathered, that the 2008 population was trending towards being significantly less infected, since the observed infected value was less than half of the expected value, though again, because it was so small, that could have changed quickly. One more netting session had been scheduled at one point, but it was raining on that day and mist-netting cannot be done in the rain, because it raises bird mortality, among many other reasons. Regardless of whether more samples were needed, others were intent on finishing the mist-netting on the day that it was ended, and circumstances were beyond control. Obviously one of the best ways to move forward with this experiment is simply to gather more data, and it was my hope that this study could be a jumping off point for future studies of the same area in the future.

The most important shortcoming of this study and in fact of the study that this one is based off of is the reliance on mist-netting to catch birds. Because mist-nets are a passive form of census, certain species are overrepresented, such as American Redstarts, and some are omitted entirely, such as the Scarlet Tanager that was seen above one net. This is because birds that generally fly high up in the air will not come in contact with the mist-nets, and birds that generally fly low to the ground will contact them much more frequently. In addition, some birds may be better equipped than others to spot the mist-nets and stay out of them, or to hit them but escape anyway, as one American Redstart was observed to have done. These factors combine to make this an imperfect method of gathering a representative blood sample population, however, the conclusions made from this data are not entirely inaccurate, especially since our main conclusion was that we could not disprove the null, and it should be noted that other than the Scarlet Tanager and some Eastern Wood-Pewees, no other passerines were observed that were not caught at all.

Yet another concern about this study is that the previous study began catching birds in May, whereas this study started in late July. As Cosgrove *et al.* (2008) noted, there is considerable seasonable variability in malaria prevalence, so any difference that is detected may be as a result of that seasonal difference, rather than a difference between years. Ideally the studies would have been performed at the same time, but that circumstance was beyond control by the middle of July.

One further concern was raised by Jarvi *et al.* (2002), when they noticed that microscopy techniques fail to accurately identify all infected birds, and only identify those with moderate levels that are found in the blood. Because malaria is a parasite that can go into the liver and entirely leave the blood stream, an infected bird may show no signs in its blood. In addition, they showed that even the PCR techniques that were used by Feldman *et al.* did not do a sufficient job of identifying all infected birds, identifying only 61-84%, though still more than the 27% of microscopy. They then showed that their new serological methods were capable of 97% accuracy among hosts experimentally infected with *Plasmodium relictum*. This finding shows new light on the inaccuracy of the microscopy techniques that both this study and its predecessor used to identify malaria, however, the conclusion that the data obtained are insufficient to reject the null hypothesis remains, since the data should be consistent when obtained by the same method. This does mean that the malaria confidence interval estimate of 5.36% to 13.36% is likely far too low and should be modified. The corrected data, using the 27% estimation from Jarvi *et al.* (2002) yielded a confidence interval of 28.12% to 41.21%. This confidence interval, calculated using variables that were uncertain, such as the actual percentage of infected birds identified with microscopy, is of less confidence than that of normal data, and therefore ought not to be trusted entirely, though as a general guide, should be fairly accurate. Future study should be directed towards the Maple River site, using PCR techniques instead of microscopy, to determine just how much our study missed and the true malaria prevalence in passerines at the Maple River mist-netting site.

Literature Cited:

Jarvi, S. I., J. J. Schultz, and C. T. Atkinson. 2002. PCR diagnostics underestimate the prevalence of avian malaria (*Plasmodium relictum*) in experimentally-infected passerines. The Journal of Parasitology 88(1):153-158

Feldman, R. A., L. A. Freed, and R. L. Cann. 1995. A PCR test for avian malaria in Hawaiian birds. Molecular Ecology 4(6):663-673.

Cosgrove, C. L., M. J. Wood, K. P. Day, and B. C. Sheldon. 2008. Seasonal variation in Plasmodium prevalence in a population of blue tits *Cyanistes caeruleus*. Journal of Animal Ecology 77(3):540-548.

Valkiunas, G. 2004. Avian malaria parasites and other Haemosporidia. CRC Press, Boca Raton.

Beadell, J. S., F. Istiaq, R. Covas, M. Melo, H. H. Warren, C. T. Atkinson, S. Bensch, G. R. Graves, Y. V. Jhala, M. A. Peirce, A. R. Rahmani, D. M. Fonseca, and R. C. Fleischer. 2006. Global phylogeographic limits of Hawaii's avian malaria. Proceedings of the Royal Society B: Biological Sciences 273(1604):2935–2944.

Mixson, S, Nguyen Y., and Yamakawa Y. 2006. Prevalence of haemosporidians in birds from northern Michigan.