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Plasmodium Prevalence in Northern Cardinals Over An Eight Month Period

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by

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Abstract

This study implemented microscopic assays on field collected data to assess the prevalence of avian malaria in a non-migratory species, *Cardinalis cardinalis.* Following capture, blood smears were collected from birds at the University of Southern Mississippi's Lake Thoreau Environmental Education and Research Center over an eight month period from September 2012 to March 2013. These smears were then stained using a Hema 3 stain set and microscopically assessed for the presence of *Plasmodium relictum* (avian malaria). Stained blood smear samples from a migrant species, *Junco hyemalis*, were used for comparisons of malarial infection. Additionally, corticosterone (CORT) assays were preformed on selected blood samples from cardinals from each seasonal time period using Arbor Assays CORT kits to determine both baseline CORT and level changes due to handling stress and season of capture and whether there was an impact of CORT on malarial infection. Of 63 *Cardinalis cardinalis* blood smear samples, none were found to have the *Plasmodium* parasite present. Similarly, all 19 *Junco hyemalis* blood smears showed no presence of the infection. On average, baseline CORT was much lower in spring than winter, but there was a more elaborate stress response in the spring. Additionally, CORT baselines and stress response levels seem to have no effect on the presence of *Plasmodium* parasites. This data will allow for further research

into *Plasmodium* overwintering behaviors along with removing *Cardinalis cardinalis* from the list of potential overwintering reservoirs.

Key Words: *Cardinalis cardinalis*, Northern Cardinal, avian malaria, *Plasmodium*

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Chapter 1: Problem Statement

Plasmodium relictum is one of the most implicated protozoan parasites in the pathogenesis of avian malaria. While some bird species are highly susceptible to this disease, others have proven to be resistant to infection, or to act as asymptomatic carriers. Avian malaria has a tendency to be lethal to bird species who have no innate immune resistance, as in the case of native Hawaii bird species where the malarial protozoa was introduced via human introduction of house mosquitoes (*Culex quinquefasciatus)* to the islands and where it has had a devastating effect on the native bird species. *Plasmodium* must be transferred via blood-sucking arthropod vectors such as the southern house mosquito, *Culex quinquefasciatus*. *Culex quinquefasciatus* is found in tropical regions and in the temperate regions of the southern United States, overlapping in may areas with the Northern Cardinal, *Cardinalis cardinalis* (Barr, 1957*).*

Because *Plasmodium* must be transferred from an infected host to a mosquito, and then back to an avian host, infected birds must be present for transmission. Species that migrate to the southern United States during the spring have been shown to carry the *Plasmodium* parasite with them, making it plausible that resident birds would be exposed and infected at this time (Garvin et al., 2006). Infected resident birds (e.g., non-migratory species) may then act as possible reservoirs for the *Plasmodium* during drops in vector

populations in the fall and winter months, allowing for the parasite to overwinter in the resident avian hosts.

High corticosterone (CORT) baselines have been linked to immunosuppression and illness in many species. Cardinals artificially implanted with CORT, taking them to levels that mimic those seen in highly stressed individuals, showed higher death rates when exposed to West Nile Virus infection (Owen et al., 2012). Birds with higher baseline CORT may therefore be more susceptible to the malaria parasite and its deleterious effects and may be more likely to serve as overwintering reservoirs.

The purpose of this research is to gain more understanding about the *Plasmodium* overwintering behavior and what role resident birds play, if any, as reservoirs. This data will help illuminate spontaneous malaria outbreaks and has implications for species conservation (Fallon et al., 2004).

Chapter 2: Literature Review

Vector Distribution and Life History

Culex quinquefasciatus, commonly called the southern house mosquito, populates the sub-tropical regions of the world between 36˚N and 36˚S latitudes. This includes the continents of North and South America, Asia, and Africa. United States distributions include most of the southern states and most recently, Hawaii (Barr, 1957, Fig. 1). This distribution overlaps with Northern Cardinal distribution in the lower United States.

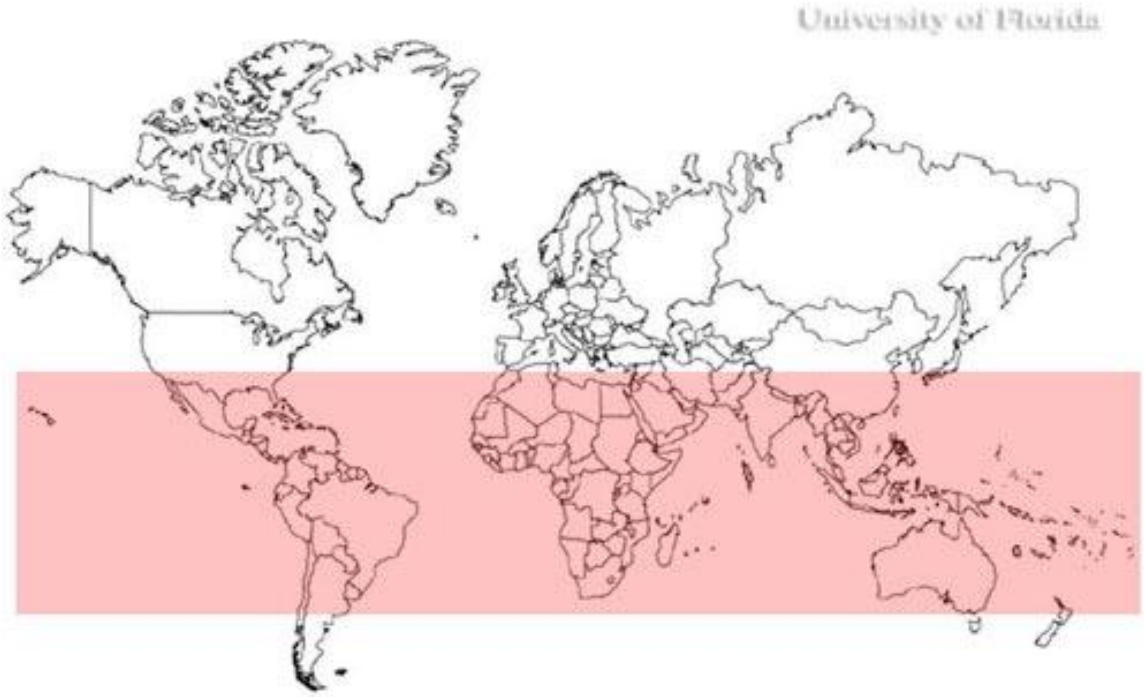

Figure 1: *Culex quinquefasciatus* distribution. (Stephanie Hill, University of Florida)

Culex quinquefasciatus larvae require temperatures of 30˚C to develop into a pupal stage and a temperature of 27˚C to move from pupa to adult (Gerberg et al., 1994). If these requirements are not met, eggs may take longer to develop or die before hatching. Female mosquitoes must take multiple blood meals after mating for egg development. Females are able to produce upwards of four egg rafts in their lifetime, depending on temperatures and standing water availability (Gerberg et al., 1994). *Culex quinquefasciatus* has been identified as a potential vector for the avian malaria parasite, *Plasmodium relictum* (Cornel et al., 2002).

Plasmodium relictum and Avian Malaria

Plasmodium relictum is a parasitic protist that is thought to have originated in American Samoa due to its chronic prevalence and cohabitation with the native land birds there. *Plasmodium relictum* is, in most cases, not fatal to the resident birds in Samoa, suggesting acquired tolerance in these birds due to long term exposer (Jarvi et al., 2003). However, when the parasite is introduced to bird populations that have no prior exposer, it can prove fatal. *Plasmodium* and avian malaria is implicated in the extinction of nearly half of the resident avian species in Hawaii (Atkinson and van Riper, 1991). *Plasmodium* is now widespread, stretching from North America to New Zealand, however both Guam and the Cook Islands have no known presence of the parasite and no cases of avian malaria (Savidge, 1985; Steadman et al. 1990; Tompkins and Gleeson, 2006).

Plasmodium can also be spread by the introduction of non-native birds into game preserves, zoos, and by species kept as pets.

Signs and symptomology of avian malaria begin when parasite presence in the bird is high. Because of this, birds may be positive for the infection, but show no symptoms due to low parasite loads (Campbell and Ellis, 2007). There is also discrepancy between species and susceptibility. Birds who have absolutely no evolutionary defense against malaria are at high risk for death, such as the endemic Hawaiian birds (Atkinson et al., 1995). This is linked to the isolation of the Hawaiian island system from any *Plasmodium* parasites (Freed et al., 2005). Damage from the disease is caused by destruction of erythrocytes (red blood cells) by the parasite which infiltrates them during development. Symptomology includes anemia, weakness due to oxygen deprivation, anorexia, hemoglobinuria, biliverdinuria, and death.

Asymptomatic carrier birds make perfect disease reservoirs and help facilitate chronic, widespread illness in less tolerant bird species (Freed et al., 2005). Birds that have been in close contact with chronic *Plasmodium* exposure seem to be able to tolerate the parasite with no overt pathogenic effects. But bird species like canaries, penguins, and domestic poultry are greatly affected by the parasite, showing high mortality rates when exposed (Campbell and Ellis, 2007).

Plasmodium can reproduce asexually while in a vertebrate host, allowing for it to multiply in the reservoir species and facilitate exposure to vectors. Sexual reproduction, however, must occur in the mosquito vector, making this stage critical for the continuation of the parasite (Glaizot et al., 2012). As with most intracellular parasites, *Plasmodium* is only infectious during the immature, motile life stage. During this phase, the cell is referred to as a sporozoite. It is at this point that it is transferred to an avian host from a vector while the vector mosquito takes a blood meal. Sporozoites enter the erythrocytes and feed off of them, using the erythrocytes to continue development into a mature schizont, or an organism undergoing replication of organelles. During this time, the parasite may completely displace the nucleus of the hosts' cells. When developed, the schizont ruptures the blood cell to release both merozoites and gametocytes. The merozoites are not motile and use the hosts' blood stream to colonize and reproduce in tissues asexually, while the gametocytes remain in the blood stream to await being ingested by a mosquito to start the process again (Campbell and Ellis, 2007).

Detection and Identification of *Plasmodium*

Microscopic assay is used to detect the presence of *Plasmodium*. Blood samples are made into smears to allow for easy viewing. Normal bird red blood cells have a uniform shape and size, though polychromatic blood cells are on average smaller than normal blood cells. The nucleus of a normal avian red blood cell may vary in location but

they are generally mononucleated and situated toward the center. All life stages of *Plasmodium* can be seen on a stained blood smear. Gametocytes are irregularly shaped and may displace the nucleus of the cell all the way to the membrane (Campbell and Ellis, 2007). Identification of the species of *Plasmodium* can be determined by looking at the appearance and location of the schizonts (Soulsby, 1982).

Corticosterone

Corticosterone is a 21-carbon glucocorticoid involved in the regulation of stress response and immune action in birds (reviewed in Adkins-Regan, 2005). Exposure to stressors (e.g., predators, starvation, inclement weather) cause a sharp spike in CORT levels allowing for the redirection of energy into life-saving responses (e.g., running, cessation of reproduction), including a drop in immune action (reviewed in Romero et al., 2009). This may lead to a lowered ability to fight off infection, especially if the stress is chronic (Apanius, 1998; Romero et al., 2009). This heightened chance of infection can lead to the organism becoming a reservoir for a pathogen. In a study using Northern Cardinals, higher CORT levels were found to be related to high morbidity rates for birds with West Nile Virus infection (Owen et al., 2012).

Here work will investigate whether cardinals show evidence of malarial infections during the non-breeding season and whether infections with malaria are linked to circulating levels of CORT. Cardinals are a resident species in the United States,

including the southeast (Halkin and Linville, 1999), they are exposed to *Culex quinquefasciatus*, and as such they can be exposed to avian malaria and act as overwintering reservoirs for the malarial parasite when vector populations are reduced due to lowered temperatures. Due to the potentially negative impacts of CORT on the immune system an assessment of CORT levels and infection status is included to determine if this is a factor in reservoir infection.

Chapter 3: Methodology

Capture

The methods to capture cardinals used here vary from breeding to non-breeding seasons. During the non-breeding season (October to February) food baited mist nets and walk-in traps are utilized to capture birds. Food provided is a general bird-feeder seed mixture. Mist nets and walk-in traps are set and open from dawn until 10:00 A.M. In breeding months (March to September), mist nets are set up at nest sites. Previous feeding watches and observations of nesting behavior influence net placement and timing of capture (done under shady or low light conditions). Cardinals are then captured while feeding nestlings. Permission to capture, band, and bleed birds is granted to our lab from the United States Fish and Wildlife Service (USFWS) Bird Banding lab (# 23479, J.M. Jawor permit holder) and the University of Southern Mississippi's Institutional Animal Care and Use Committee (IACUC, #s 10081204, 10081203, and 11092214).

Once captured, unbanded birds are fitted with USFWS numbered bands and previously banded birds are identified by number. Physical measurements and body condition are recorded for each bird. These measurements include mass (g), tarsus length (mm), and wing cord length (mm). Blood is then collected from the ulnar wing vein via venipuncture with a 25 gauge needle and collected with a heparinized microhematocrit capillary tube. Blood smears are created using the push-slide method, which requires a small drop of blood be placed at the end of a microscope slide while using another slide held at a 30˚ angle to spread the blood in a monolayer film (Schalm et al., 1975). The blood smears are then stored in a dry container until staining. During this work 63 blood smears were collected.

Staining and Microscopic Assay

The blood smears were stained using a Hema 3 stain set (Fisher Diagnostics, Fisher Scientific Company L.C.C., Middletown, VA, United States) which included using the Hema 3 Fixative Solution and dyes to both preserve and visualize red and white blood cells. The smears from the cardinals and the smears from Dark-eyed Juncos (*Junco hyemalis*, caught previously in winter 2008-2009 and used as a comparison set) were stained. Work with Dark-eyed Juncos is also covered by permits and IACUC permission (see above) held by J.M. Jawor. No juncos were captured during this work and previous work done with juncos is not described here (different focus entirely); although capture, handling, and bleeding techniques are identical to those used for cardinals. After staining, the smears were allowed to air dry before examination. Slides were then examined under X100 oil immersion optics using a Zeiss Axiostar plus microscope. Numbers of malarial parasites are reported as parasites observed out of 100 red blood cells assessed per slide.

Corticosterone Analyses

Corticosterone levels were assessed from a sub-set of individuals using an enzyme immunoassay (EIA; Arbor Assays, LLC, Ann Arbor, MI, U.S.A. No. K014-H5) using techniques previously described for cardinals (DeVries and Jawor, *in press*). Briefly, 20 µl of radiolabelled CORT was added to all plasma samples (10 µl of plasma used) to determine extraction efficiency. Triple extractions with diethyl ether were preformed and extracts were re-suspended with the provided assay buffer (brought to 400 µl). To perform the assay, duplicate 50µl aliquots were taken from each re-suspended sample, placed in adjacent EIA plate wells, exposed to kit-supplied antibodies that bind to CORT and last exposed to kit-supplied color-changing substrate (changes the color of

CORT-antibody complexes in the wells). Change in color in the wells corresponds to CORT amount in the extracted sample. Plates are read on a BioRad Model 680 microplate reader at 450 nm and readings are compared to a seven point standard curve to determine CORT concentrations. To determine variation within the EIA assay plate itself samples from a uniform plasma pool were used. Following plasma extraction the average extraction efficiency was determined to be 82.7% (determined from a single 100 µl aliquot drawn from the extracted, reconstituted plasma), intra-assay variation was determined to be 17% and as only a single plate was run there is no inter-assay variation that needed to be accounted. Statistical analyses were completed using IBM SPSS Statistics Version 20.

Chapter 4: Results

Blood smears were separated into fall, winter, and spring sets. The fall set contained samples collected from September to November and was comprised of 10 females and 18 males for a total of 28 samples. The winter set contained samples collected from December to February and contained 8 females and 19 males for a total of 27 samples. The spring set contained samples collected from March to April and was comprised of 3 females and 5 males for a total of 8 samples. Of the 63 total samples, none showed the presence of *Plasmodium*.

To help determine whether cardinals are resistant to the parasite, or the parasite isn't prevalent in birds during the non-breeding season in the southeastern United States, blood smears from overwintering Dark-eyed Juncos were also assessed. The 19 Dark-eyed Junco winter blood smears used for comparison also had no *Plasmodium* present.

Since there were no observed *Plasmodium* infections in Northern Cardinals in this study we were not able to compare *Plasmodium* to CORT, however we did assess CORT levels. A paired samples T-test was performed to determine significance of CORT level changes before and after handling during banding and bleeding. Results show a significant change ($t=3.88$, $df=14$, $P=0.002$, Fig. 2) in CORT levels from pre- and post-handling blood samples. The fall average baseline CORT level was 39.7 ng/ml while the average stress response was 54.8 ng/ml. The winter CORT baseline was 40.064ng/ml with an average stress response at 78.1 ng/ml. Spring CORT baselines was 16.6 ng/ml with and average stress response of 85.7 ng/ml.

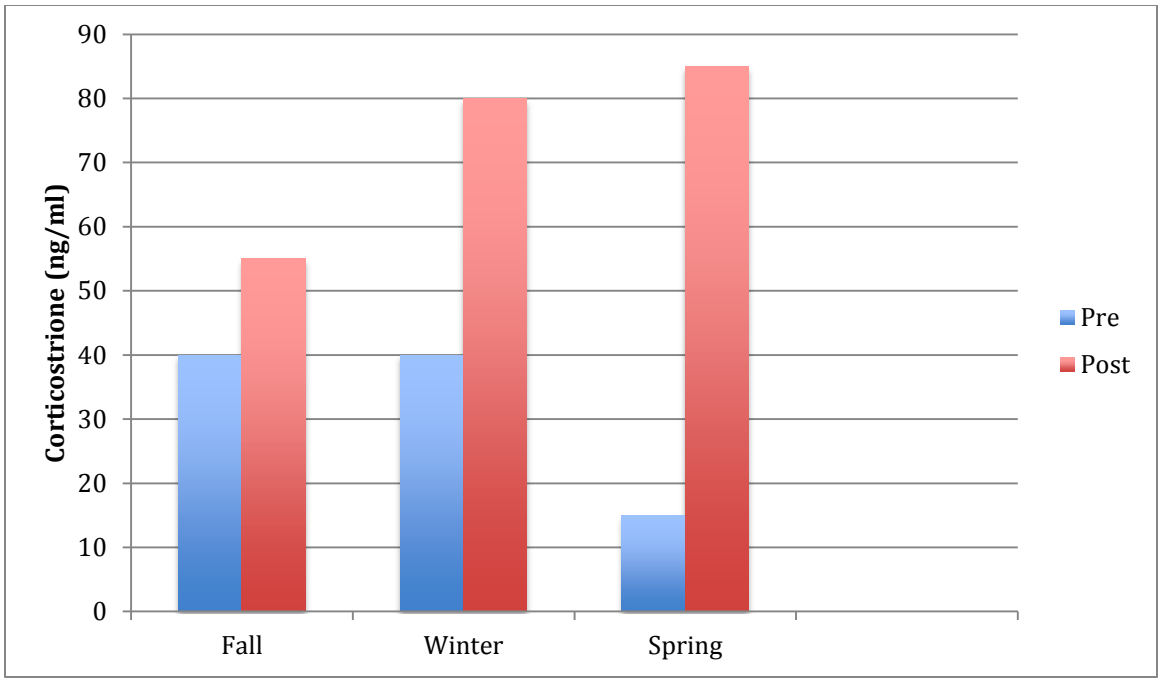

Figure 2. Corticosterone pre- and post- handling levels by season.

Chapter 5: Discussion

The lack of *Plasmodium* in the Northern Cardinal blood samples suggests that cardinals are not used as a significant overwintering reservoir for this parasitic species. The presence of both the vector and bird species that are known to have the *Plasmodium* parasite eliminates the possibility of this disease just not being in the study area (Garvin et al., 2006). Rather it suggests that cardinals may not be as susceptible to infection with this parasite or they do not experience significant reproduction of this parasite in their bodies over the winter.

The Dark-eyed Junco blood smears used for comparison as a species also found in the study area during the winter months, showed no signs of *Plasmodium* infection. This may mean that *Plasmodium* is not as prevalent in the winter months due to low populations of mosquitos breeding and therefore partaking in a blood meal. Potentially, infection with *Plasmodium* over the winter may be unlikely for any bird species. However, there is still evidence that cardinals may be slightly more resistant to infections with *Plasmodium.* While the junco smears were collected in only the winter (the only time juncos are found in the southeastern United States) the cardinal smears encompassed three seasons for collection. Mosquitos are still quite active and populous during September and October and even cardinals surveyed in these months did not show evidence of malarial infection. Further assessment in the summer, the period of most intense breeding behavior and hence energy expenditure and stress, is also needed to confirm low susceptibility in cardinals to the malarial parasite.

Corticosterone baseline levels, and the level of stress response achieved, seemed to play no role in the appearance of the parasite in cardinals, as birds with higher

baselines (winter and fall) and birds with low baselines (spring) had no parasites present. Birds that mounted a particularly acute stress response were also not likely to have malarial parasites, regardless of season. While not a part of this research it is noteworthy that stress responses in cardinals were becoming more elaborate as spring approached. If this pattern of stress response continues into the breeding season individuals may change in their susceptibility to malaria and the ability of the parasite to amplify in their bodies. Additionally, the lack of parasite infection may change when the birds are chronically stressed regardless of season (as in Owen et al., 2012) and individuals have overall poorer energy stores (Romero et al., 2009).

As *Plasmodium* wasn't found in currently active species at the study site (resident cardinals and wintering, migrant juncos) but it is known to occur in the southeastern United States a logical question is how does this parasite either overwinter or return each year? Migrant species such as the Red-eyed Vireo (*Vireo olivaceus)*, Swainson's Thrush (*Catharus ustulatus)*, Summer Tanager (*Piranga rubra)*, and Orchard Orioles (*Icterus spurius*) have all been shown to carry *Plasmodium* back with them during the spring migration and all of these species breed in the southeastern United States (Garvin et al., 2006). This potentially provides a source of malarial parasite reintroduction to the host (resident and migrant) avian populations in the southeastern United States. As the parasite may not be overwintering in resident birds in the area, this could mean that it is being repeatedly reintroduced by infected individuals when they migrate from the southern hemisphere for the breeding season, and that residents either are not (or are less) susceptible to overwinter infection, or they clear the parasite from their systems prior to winter occurring.

For future research, testing other non-migratory bird species in the area for the same parasite should be considered. This will help eliminate the possibility that cardinals are somehow immune to the infection, or that they are clearing it from their bodies too quickly for it to have a health impact or amplify. The parasite is obviously not endemic to the area and the species that inhabit it. Endemism is typically characterized by chronic observable, infections and a prevalence of asymptomatic avian carriers. It is most certainly found in the study area and it is likely being brought into the area by infected birds. With the vectors present and migrant birds acting as short-term reservoirs, it is reasonable to assume that the parasite is being reintroduced with frequency.

The possibility of a non-avian reservoir for overwintering should also be considered. Mosquito eggs in diapause (a situation where development is stopped until appropriate temperatures are experienced) could potentially protect the parasite until conditions are right for the arthropod host to break overwintering diapause and receive its blood meals. This may allow for the development of the *Plasmodium* parasite population each spring and for the persistence of the parasite in the population over each winter.

Overall, this study showed that Northern Cardinals do not act as significant reservoirs for the *Plasmodium* parasite from the fall to the spring. While the parasite is present in birds that breed in the southern United States and even though there are proper vectors for parasite transmission in the summer and winter, there were no blood smears that showed infection. Corticosterone levels were also ruled out as a contributing fact to presence of *Plasmodium*, as both birds with high and low levels of CORT showed no infection. Future studies should address whether other species serve as overwintering

reservoirs, whether migrating birds move malaria into the area annually, or whether there are other mechanisms for this parasite to overwinter.

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Literature Cited

Atkinson, C. T., Woods, K. L., Dusek, R. J., Sileo, L. S. and Iko, K. W. 1995. Wildlife disease and conservation in Hawaii: pathogenicity of avian malaria (*Plasmodium relictum*) in experimentally infected I'iwi (*Vestiaria coccinea*). Parasitology 111 Suppl.: S59–S69.

Adkins-Regan E. 2005. *Hormones and Animal Social Behavior*. Princeton, New Jersey: Princeton University Press.

Apanius V. Stress and immune defense. Adv Study Behav 1998, 27:133-153.

Barr AR. 1957. The distribution of *Culex p. pipiens* and *C. p. quinquefasciatus* in North America. J Tropical Med Hygiene 6: 153-165.

Campbell T and Ellis C. 2007. *Avian and exotic animal hematology and cytology*. (3rd ed.). Ames, Iowa: Blackwell Publishing Professional.

Cornel AJ, Mcabee RD, Rasgon J, Stanich MA, Scott TW and Coetzee M. 2002. Differences in Extent of Genetic Introgression Between Sympatric *Culex pipiens* and *Culex quinquefasciatus* (Diptera: Culicidae) in California and South Africa, J Med Entomology 40(1).

DeVries MS and Jawor JM. Natural variation in circulating testosterone does not predict nestling provisioning rates in the northern cardinal, *Cardinalis cardinalis*. Anim Behav, *In press*.

Fallon SM, Ricklefs RE, Latta SC, and Bermingham E. 2004 *Temporal stability of insular avian malarial parasite communities*. Proc Biol Sci 271:493-500.

Freed LA, Cann RL, Goff ML, Kuntz1 WA, and Bodner GR. 2005. Increase in Avian Malaria at Upper Elevation in Hawaii. Condor 107:753–764.

Garvin M, Szell C, and Moore F. 2006. Blood parasites of neartic-neotropical migrant passerine birds during spring trans-gulf migration: Impact on host body condition. J Parasitology, 92:990-996.

Gerberg EJ, Barnard DR, and Ward RA. 1994. Manual for Mosquito Rearing and Experimental Techniques. American Mosquito Control Association Bulletin No. 5:61-62.

Gleeson D, and Tompkins D. Manaaki Landcare Research. *Avian malaria in New Zealand-Hawaii all over again?* Centre for Biodiversity and Biosecurity Seminar Series, University of Aukland.

Halkin SL and Linville SU. 1999. Northern cardinal (*Cardinalis cardinalis*), N. 440. In The Birds of North America (Ed. by A. Poole). Ithaca, New York: Cornell Lab of Ornithology.

Jarvi SI, Farias MEM, Baker H, Freifeld HB, Baker PE, Gelder EV, Massey G, and Atkinson CT. 2003. Detection of avian malaria (*Plasmodium* spp.) in native land birds of American Samoa. Conservation Genetics 4:629-637.

Owen J, Nakamura A, Coon C, and Martin L. 2012. The effect of exogenous corticosterone on West Nile virus infection in northern cardinals. Vet Research 43:34.

Romero LM, Dickens MJ, Cyr NE. 2009. The reactive scope model: a new model integrating homeostasis, allostasis, and stress. Horm Behav 55:375-389.

Savidge JA. 1985. The role of disease and predation in the decline of Guam's avifauna. PhD dissertation, University of Illinois, Urbana.

Schalm OW, Jain NC, and Carroll EJ. 1975. Veterinary hematology. Vol 3, *Veterinary Hematology*, edited by O. Schalm, N. Jain, and E. Carroll. Philadelphia: Lea and Febiger.

Steadman DW, Geiner EC and Wood CS. 1990. Absence of blood parasites in indigenous and introduced birds from the Cook Islands, South Pacific. Conservation Biol 4:398–404.

Tompkins DM, and Gleeson DM. 2006. Relationship between avian malaria distribution and an exotic invasive mosquito in New Zealand. J R Soc of New Zealand 36(2): 51-62.

Van Riper, III CS. 1991. The impact of introduced vectors and avian malaria on insular passeriform bird populations in Hawaii. Bull Soc Vector Ecol 16:59–83.