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Incidence of Avian Malaria on Wild Birds in Humid Premontane Forest of Pichincha Province in Ecuador

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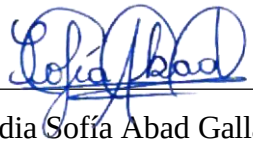

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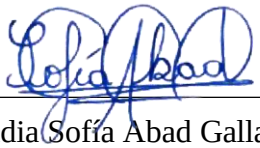

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DEDICATORY

This work is dearly dedicated to my parents, who always encouraged me to pursue my dreams and aspirations, and helped me in more than one way to accomplish this one. It is also dedicated to my family; I am extremely grateful for your motivation and accompaniment throughout my university journey. Thank you, as well, for your support and strength when things were not easy; you all were the mainstay that made the completion of this work possible.

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ABSTRACT

Avian malaria is a tropical zoonotic disease caused by protozoans of the genera *Plasmodium* and *Haemoproteus*, belonging to the phylum Apicomplexa. Parasites in these genera can affect several vertebrates like mammals, reptiles, and birds being species-specific to each class. Avian malaria is not a lethal disease, but it affects birds' lifespan along with their reproductive rate, which exerts at once a direct effect on birds' biodiversity. In addition, there are only few studies of avian malaria in Latin American, Ecuador included, with some reports from the Galapagos Islands; therefore, the studied areas are of interest.

The presence of *Plasmodium* in hummingbirds and passerines was investigated in two humid premontane forest areas managed by the Mindo Cloudforest Foundation (MCF); near Nanegalito (Puyucunapi Pilot Project) and Milpe (Milpe Bird Sanctuary). Prevalence and parasitemia were determined by microscopic examination of blood smears and stained with the Giemsa's reagent. However, for more accurate identification and differentiation between *Plasmodium* and *Haemoproteus*, serological and molecular methods will be performed as well in the near future. Both study sites are part of a 1000 m elevation gradient; hence, elevation was used as a predictor variable for prevalence and parasitemia levels in a Mann-Whitney U test. This test was also used to test for a sex bias.

This study reports a total of 21 bird species that inhabit both humid forest localities. The Milpe area, which represents the less elevated of the two examined sites, has a prevalence of 100%, while the Nanegalito area presents a prevalence of 96%. The statistical analysis indicates that there is not enough evidence to claim an elevational difference in prevalence and parasitemia. Furthermore, by comparing the results from this work to the data of Spencer et al. in the inter-Andean dry forest of Imbabura Province, it was found that maximum parasitemia levels were similar with that of 9% in northwestern Pichincha and 8% in the warmer and less elevated site studied in the tropical dry forest. Prevalence depended to a small degree on elevation. On average, the humid forest has a total prevalence of 97% while the dry forest of 80%, showing that elevation along with several eco-geographical features influence the infection degree in birds since they affect the proliferation of the insect-vector.

Keywords: Avian malaria, Pichincha, humid forest, *Plasmodium*, and *Haemoproteus*.

RESUMEN

La malaria aviar es una enfermedad zoonótica tropical causada por protozoos de los géneros *Plasmodium* y *Haemoproteus*, pertenecientes al filo Apicomplexa. La malaria puede afectar a otros vertebrados como mamíferos y reptiles, siendo la infección específica para cada clase. La malaria aviar no es una enfermedad letal pero afecta el tiempo de vida de las aves junto con su tasa de reproducción, lo que a su vez ejerce un efecto directo sobre la biodiversidad de las aves. Además, existen pocos estudios de malaria aviar en América Latina, incluido Ecuador, con algunos reportes en las Islas Galápagos; por tanto, las áreas estudiadas en este trabajo son de interés.

La presencia de *Plasmodium* en colibríes y aves paseriformes se investigó en dos lugares de bosque húmedo premontano administrados por la Fundación Mindo Cloudforest Foundation (MCF); las áreas de Puyucunapi y Milpe. La prevalencia y la parasitemia se determinaron mediante examen microscópico de frotis de sangre y se tiñeron con el reactivo de Giemsa. Sin embargo, para una identificación y diferenciación más precisa entre *Plasmodium* y *Haemoproteus* también realizarán métodos serológicos y moleculares en un futuro próximo. Los dos sitios forman parte de una gradiente altitudinal en las estribaciones del volcán Pichincha. Por lo tanto, una prueba U de Mann Whitney fue utilizada para determinar si había diferencias en parasitemia y prevalencia debido a elevación de los sitios de estudio y otra para determinar si había un sesgo debido a sexo en los datos.

Este estudio reporta un total de 21 especies de aves que habitan ambas localidades de bosque húmedo. El área de Milpe, que representa el menos elevado de los dos sitios examinados, tiene una prevalencia del 100% mientras que el área de Nanegalito presenta una prevalencia del 96%. Los resultados del análisis estadístico indican que no existe evidencia suficiente para afirmar que la diferencia altitudinal es el parámetro responsable de los resultados obtenidos. Adicionalmente, al comparar los resultados de este trabajo con los datos proporcionados por el grupo de investigación de la Dra. Spencer et al. en el bosque seco interandino de la provincia de Imbabura, se encontró que los niveles de parasitemia máxima eran similares con 9% en bosque húmedo y 8% en bosque seco interandino. Finalmente, el bosque húmedo tiene una prevalencia total del 97% mientras que el bosque seco del 80%, lo que demuestra que la elevación junto con varias características eco-geográficas influyen en el grado de infección en las aves ya que afectan la proliferación del insecto-vector.

Palabras Clave: Malaria aviar, Pichincha, bosque húmedo, *Plasmodium* y *Haemoproteus*.

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1. TITLE

Incidence of Avian Malaria on Wild Birds in Humid Premontane Forest of Pichincha Province in Ecuador.

2. INTRODUCTION AND JUSTIFICATION

Parasitism is a pervasive species interaction around the globe, with over 31,000 protozoan parasites and more than three-quarters yet to be reported. The research that has been conducted so far on protozoan species, is usually linked with their medical and economic significance. Species parasitizing mammals have been more widely studied than those affecting birds and reptiles. (Matta & Rodríguez, 2001). Malaria is an outstanding parasitic disease, caused by protozoans of the phylum Apicomplexa, which was defined by Levine in 1970 (Escalante & Ayala, 1994). Additionally, it is a tropical zoonotic emerging infectious disease (EID) that can affect several vertebrates, and that is specific-species for each class. As a result, protozoans causing avian malaria cannot infect mammals nor reptiles. (Valkiūnas & Iezhova, 2018)

Avian malaria was discovered in 1885, and from that point on, it has played a significant role in the understanding of human malaria. Not long ago, it was also employed to explain how malaria parasites are transmitted and to develop new medication to treat the disease. Recognizing and describing avian malaria lineages in the field has led to the acknowledgment of the prevalence (defined as the proportion of infected exemplars), diversity, and distribution of malaria all over the world. Avian malaria was and still represents a perfect experimental model for researchers because bird parasites have a strong resemblance to human malaria parasites. For that reason, this model has thrived in the past 60 years and is a trending topic to research (Rivero & Gandon, 2018).

Plasmodium and *Haemoproteus* are genera of haemosporidians, blood-borne protozoan parasites, and the etiological agents of malaria in birds (Escalante & Ayala, 1994; Matta & Rodríguez, 2001). *Plasmodium* is a mosquito parasite while *Haemoproteus* is transmitted by biting midges and louse flies (Ferreira Junior et al., 2017). *Plasmodium* and *Haemoproteus* are paraphyletic; therefore, they can be confused since both share biological features because of having a common ancestor (Paul, Ariey, & Robert, 2003). For example, the gametocytes of both genera are almost indistinguishable (Matta & Rodríguez, 2001). Furthermore, malaria pathogens infect not only the blood but also several organs in birds (Valkiūnas & Iezhova, 2018).

Avian malaria has the potential to exert strong selective pressure on birds since it is frequently virulent (Rivero & Gandon, 2018). Some of the effects on birds' physiology during the initial acute phase of the infection are anemia, lethargy, and appetite loss (Vogel, 2015). Moreover, the avian malaria effects are especially calamitous when new parasite lineages are unintentionally introduced into an immunologically naïve host population that was never infected before by that specific lineage (Delhaye, Jenkins, Glaizot, & Christe, 2018; Rivero & Gandon, 2018).

The hematozoan parasites' life cycle is divided into two different phases, one that includes asexual reproduction, which occurs in the vertebrate host, and one that requires sexual reproduction in the invertebrate host's, or vector's, midgut (Martínez de la Puente et al., 2020). These parasites usually complete sporogony moderately quickly in vulnerable vectors at medium to low temperatures (Ferreira Junior et al., 2017). Thereby, the global distribution of the disease is influenced by several factors such as seasonality, eco-geographical features, elevation, and also the presence of water bodies required by the vector (Durrant et al., 2006; Ferreira Junior et al., 2017; Okanga, Cumming, & Hockey, 2013; Robinet & Roques, 2010). These protozoans have been reported in vectors even near to the Polar Circles (Valkiūnas & Iezhova, 2018). Despite being widely distributed across the globe, avian malaria parasites have similar patterns of diversity to their hosts. In general, *Haemoproteus* is more diverse than *Plasmodium* except in South America where *Plasmodium* is prevalent and widespread (Clark, Clegg, & Lima, 2014).

2.1. Problem statement

As mentioned above, parasites are cosmopolitan in distribution causing many diseases in various species, including humans (Matta & Rodríguez, 2001). Malaria is a well-known tropical parasitic disease caused by protozoans of the phylum Apicomplexa (Escalante & Ayala, 1994; Moens & Pérez-Tris, 2016; Matta & Rodríguez, 2001). The genera responsible for avian malaria are *Plasmodium* and *Haemoproteus* (Moens & Pérez-Tris, 2016). *Plasmodium* parasites is of greater interest for this study since the genus includes species of human medical concern, and because they serve as a good model to study the impact of their relatives on human health (Rivero & Gandon, 2018). Avian malaria can be detected by microscopic examination of blood smears stained with the Giemsa reagent, a technique generally used for parasitological diagnosis (Clark et al., 2014). However, for more accurate identification and differentiation between the two mentioned genera, serological and molecular methods should be performed to diagnose malaria

infections in wild birds (Fallon, Ricklefs, Swanson, & Bermingham, 2003; Hellgren, Waldenström, & Bensch, 2004). There are few studies of avian malaria in Latin American, including the Ecuadorian territory. Nevertheless, there are some reports from the Galapagos Islands (Asigau & Parker, 2018; Palmer et al., 2013). Their potential impact on biodiversity makes it important to investigate the effects of Haemosporida hemoparasites causing avian malaria on the physiology of wild birds, especially regarding reproductive success and lifespan (Eastwood et al., 2019). This study explores patterns in prevalence and parasitemia in the different species of infected birds existing in humid premontane forests which is important (Alves Mata, 2012). This preliminary study represents the first step to broaden avian malaria research in Ecuador, in order to have a better understanding of how this disease is affecting bird species biodiversity in the country.

2.2. Objectives

2.2.1. General Objective

To detect patterns in Haemosporida hemoparasites, especially *Plasmodium,* infections that can affect wild birds' reproductive success and lifespan and, in consequence, their biodiversity.

2.2.2. Specific Objectives

- To describe the levels of parasitemia in wild birds.
- To review experimental studies that analyze the effects of the infection on birds' physiology, especially within Latin America.
- To determine if the degree of infection, in both humid and dry forests, depends on the elevation.
- To compare the experimental data obtained from the two humid forest locations with the data provided by Dr. Spencer's research group from the tropical dry, inter-Andean forest to determine if the differences can be attributed to ecosystem type.
- To infer which species are at risk of extinction in both ecosystems since the infection affects the reproduction rate of bird species.

3. BIBLIOGRAPHIC REVIEW

3.1. The Biology of Avian Malaria

Malaria is a disease that affects millions of people around the globe, mainly in the tropics; it can also infect other mammals along with several reptile and bird species (Escalante & Ayala, 1994). Malaria parasites were classified in 1966 into 9 different subgenera, three of which parasitize mammals, two affect reptiles, and four infect birds (Qari, Shi, Pieniazek, Collins, & Lal, 1996). *Plasmodium* and *Haemoproteus* both cause avian malaria and belong to the phylum Apicomplexa (Figure 1). *Plasmodium* uses a mosquito parasite as vector whereas *Haemoproteus* is transmitted by biting midges and louse flies (Ferreira Junior et al., 2017).

An important aspect of the avian malaria parasites' biology is the phylogenetic relationship between them and the host birds. To understand this relationship, it is fundamental to analyze the evolutionary history of haemosporidian malaria parasites. The orders of Haemosporida includes around 200 known species of bird malaria parasites, 150 lizard parasite species, and approximately 20 species of non-primates parasites for mammals (Outlaw & Ricklefs, 2014). *Plasmodium* alone contains nearly 172 species in total, 89 affecting reptiles, 51 occurring in mammals and 32 being specific to birds. Therefore, it is important to be aware of the relationship between both genera causing avian malaria; *Plasmodium* is paraphyletic to *Haemoproteus* and can be easily confused since they share many biological features due to a common ancestor (Paul et al., 2003). One example of these similarities is the shape of their gametocytes. Indeed, they are so similar that result almost indistinguishable (Matta & Rodríguez, 2001).

The phylogeny reconstruction of Haemosporida parasites is complex, hence, incomplete. The most significant limitations for this area of study include lack of taxon and character sampling, along with biases in the composition of nucleotides (Galen et al., 2018). Regardless, current sampling and analytical techniques have revealed that the avian *Plasmodium* parasites are the sister group of *Plasmodium* infecting mammals, i.e., they are paraphyletic (Galen et al., 2018; Outlaw & Ricklefs, 2014). However, bird parasites are more diverse concerning vector diversity, levels of host specificity, and geographic range, than their mammalian counterparts (Outlaw & Ricklefs, 2014). For example, avian *Plasmodium* is transmitted by numerous mosquito species of the family Culicidae, generally corresponding to the genera *Aedes*, *Anopheles*, and *Culex*, and less commonly to *Culisetta*, *Coquillettidia*, *Mansonia*, and *Psorophora*. In contrast, human malaria is only

transmitted by female *Anopheles* mosquitos (Njabo et al., 2011; Raghavendra, Barik, Niranjan, Sharma, & Dash, 2011; Valkiūnas & Iezhova, 2018). The parasites use the dipteran host to complete sporogony and then be transmitted by mosquito vectors (Njabo et al., 2011; Valkiūnas & Iezhova, 2018).

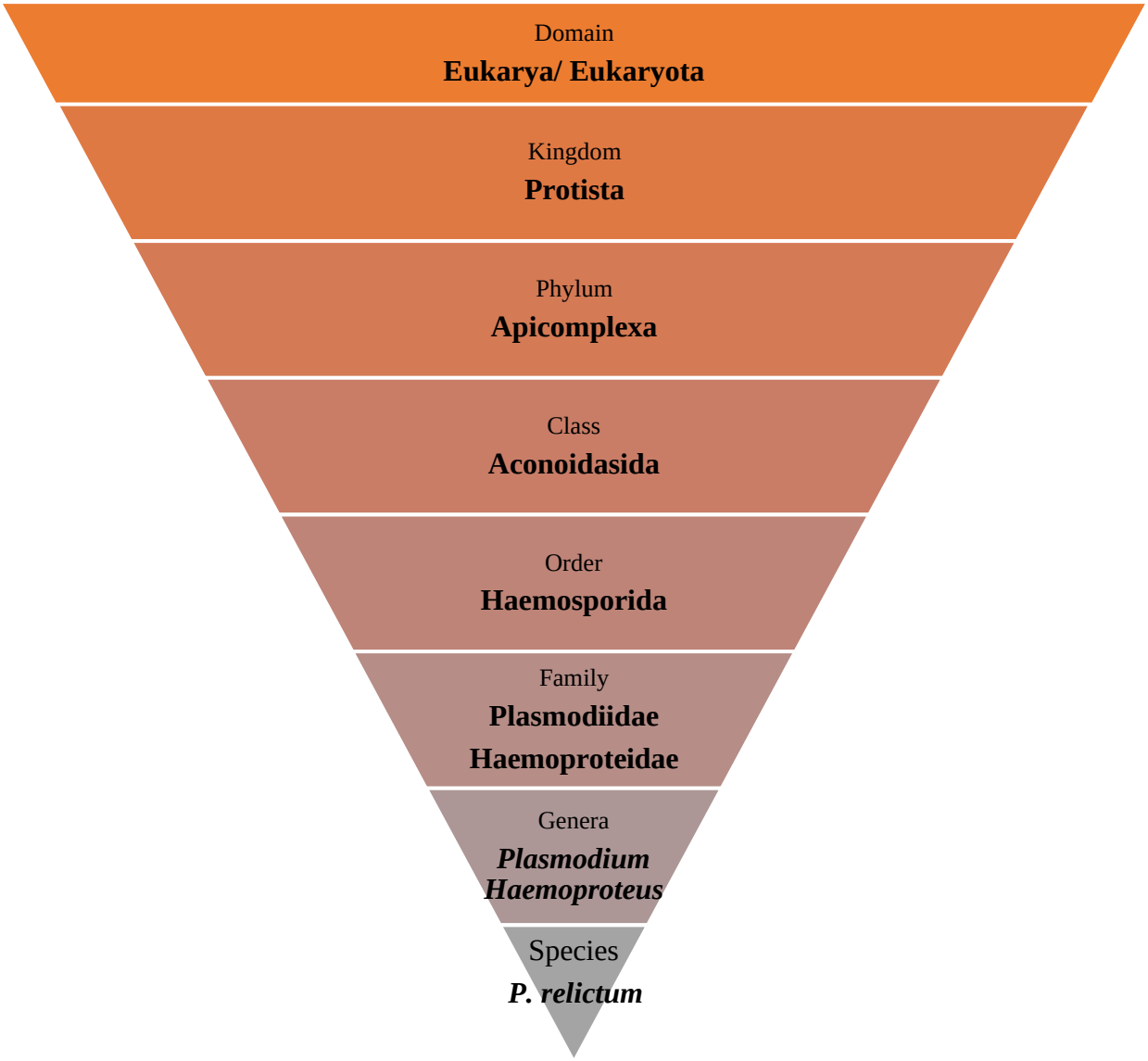

Figure 1. Avian malaria taxonomy, including the most common species to cause malaria in birds, *Plasmodium relictum*. Source: (Santiago-Alarcon, Palinauskas, & Schaefer, 2012; Valkiūnas & Iezhova, 2018)

3.1.1. Avian Malaria Life Cycle

The protozoans causing malaria are obligate heteroxenous protists, i.e., they need more than one host to complete their life cycle (Valkiūnas & Iezhova, 2018). Thus, the life cycle of malaria hemoparasites involves two hosts, the invertebrate host or insect-vector, and the vertebrate host. The general life cycle of haemosporidian parasites explains the sequence of events for both *Plasmodium* and *Haemoproteus* (Figure 2). However, there are some differences in their infection processes. The first one is that they use different vector families of the Diptera order; *Plasmodium* utilizes blood-sucking mosquitoes (Culicidae) while *Haemoproteus* employs biting midges (Ceratopogonidae) and louse flies (Hippoboscidae; Valkiūnas & Iezhova, 2018).

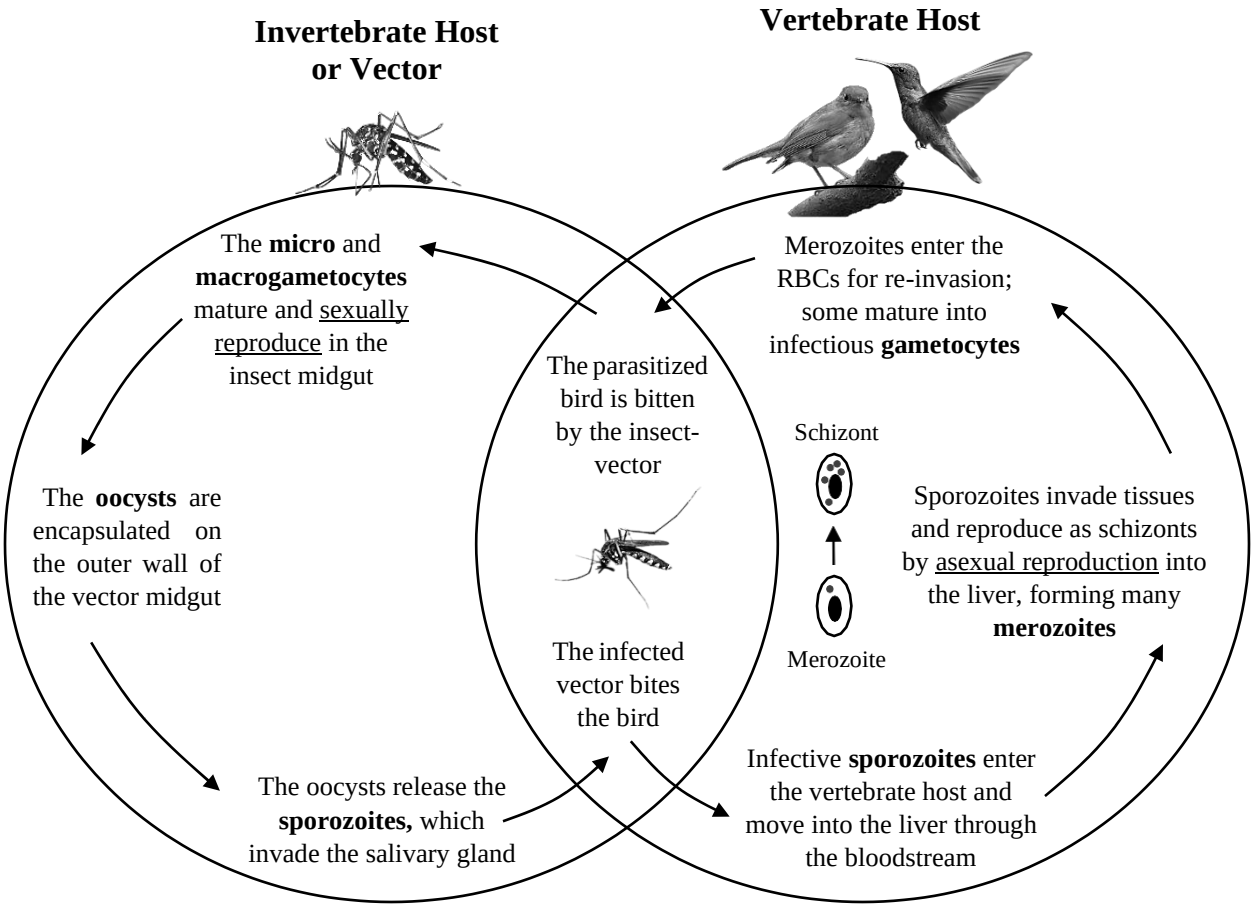

Figure 2. Haemosporidians' general life cycle. Adapted from Alves Mata, 2012, and Matta & Rodríguez, 2001.

Haemoproteus life cycle begins when the dipteran vector ingests infected blood with microgametocytes and macrogametocytes. The insect-vector is also known as the definite host because the sexual reproduction stage, or sporogony, occurs within the insect. Then, the

gametocytes mature in the midgut of the insect and, through sexual reproduction, produce mobile zygotes that penetrate the epithelial cells of the midgut giving rise to oocysts. Once the oocyst matures, it releases the sporozoites, which are the infectious parasitic form that will migrate to and accumulate in the salivary glands of the invertebrate host, making it possible to infect another vertebrate host with its next bite (Okanga et al., 2013; Matta & Rodríguez, 2001; Spencer, Mendoza, & Louro, 2016).

After the infected vector bites a bird or intermediate host, the sporozoites can infect several organs like the heart, spleen, lung, liver, kidneys, and brain (Ilgūnas et al., 2019; Palinauskas et al., 2016; Matta & Rodríguez, 2001). Then, through asexual reproduction or merogony, the merozoite passes to the schizont form. The final maturation process of the schizont is achieved with the liberation of hundreds of merozoites. After that, the merozoites can parasitize diverse organs or move into the bloodstream where some develop into gametocytes within the nucleated erythrocytes of birds (Doussang et al., 2019; Rivero & Gandon, 2018; Matta & Rodríguez, 2001). Finally, the gametocytes differentiate into microgametocytes and macrogametocytes. The infection process can have a duration between 9 to 12 months and is usually asymptomatic (Matta & Rodríguez, 2001; Sinden, 2016; Videvall, Palinauskas, Valkiūnas, & Hellgren, 2020).

On the other hand, despite *Plasmodium* and *Haemoproteus* having very similar life cycles, a significant difference is that *Haemoproteus* schizonts are only found in the internal organs of the vertebrate host while *Plasmodium* parasites can be found in the red blood cells as both sexual and asexual forms (Matta & Rodríguez, 2001). This difference is due to asexual reproduction or merogony in *Plasmodium* occurring in the peripheral blood of the vertebrate host, while in *Haemoproteus* it occurs in cells of tissues found in the previously mentioned organs (Santiago-Alarcon et al., 2012; Valkiūnas & Iezhova, 2018). Finally, these genera have a similar life cycle to *Leucocytozoon*, the third genus of avian haemosporidian parasites; however, this genus is transmitted by blackflies and does not cause malaria but other pathologies in birds (Martínez de la Puente et al., 2020; Njabo et al., 2011). Moreover, *Leucocytozoon* are not generally found in wild birds as they primarily affect poultry such as ducks and turkeys (Santiago-Alarcon et al., 2012).

3.1.2. Importance of Avian Malaria for Human Malaria Diagnosis and Treatment

Malaria transmission is a major concern for public health. Understanding it is essential for developing new strategies for its eradication (Meibalan & Marti, 2016). The global dimensions of malaria are staggering. In 2018 the World Health Organization (WHO) registered 228 million malaria cases and 405 000 malaria deaths globally (World Health Organization, n.d.). All human malaria parasites belong to the genus *Plasmodium*, with the five reported species being *P. vivax*, *P. falciparum*, *P. malariae*, *P. ovale*, and *P. knowlesi* (Escalante & Ayala, 1994; L. M. Spencer, Gómez, & Collovini, 2016; Weber, 1988). The last one is responsible for other mammal species infections as well (Sullivan et al., 2005). Moreover, *P. falciparum* is the most virulent of the five species. Some evidence is suggesting that it spilled over from birds to humans not long ago. This also would explain why this protozoan is closer to bird *Plasmodium* parasites in the phylogenetic tree than the other four species (Qari et al., 1996).

Since the discovery of avian malaria in 1885, it has played a relevant role in the understanding of human malaria because it was used as the first experimental system to explain the biology of malaria transmission. Moreover, it was employed to develop new medications to treat the disease, such as antimalarial drugs and vaccines. Recognizing and describing avian malaria lineages in the field has led to the acknowledgment of the prevalence, diversity, and global distribution of malaria. Avian malaria has been a perfect experimental model for researchers because bird parasites have a strong resemblance to human malaria parasites. For that reason, this model has thrived in the past 60 years and is still a trending topic in malaria research (Rivero & Gandon, 2018).

3.1.3. Avian Malaria Diagnostics

Nowadays, the concern over prevalence and incidence of vector-borne parasites is heightening. Hence, it is essential to correctly diagnose avian malaria pathogens and to study their effects on the physiology of infected specimens (James, 2002). One way to differentiate between *Haemoproteus* and *Plasmodium* is by observing dyed blood samples under a microscope. A significant difference between the two genera is that in *Plasmodium* sexual and asexual forms of the parasites can be found in blood cells (Figure 3) whereas in *Haemoproteus* schizonts are only found in the host's internal organs (Matta & Rodríguez, 2001). Therefore, the presence of a schizont in a bird´s blood cell confirms infection by *Plasmodium*. However, the best differentiation

methods are the polymerase chain reaction (PCR) or performing a serological analyses (Jarvi, Schultz & Atkinson, 2002).

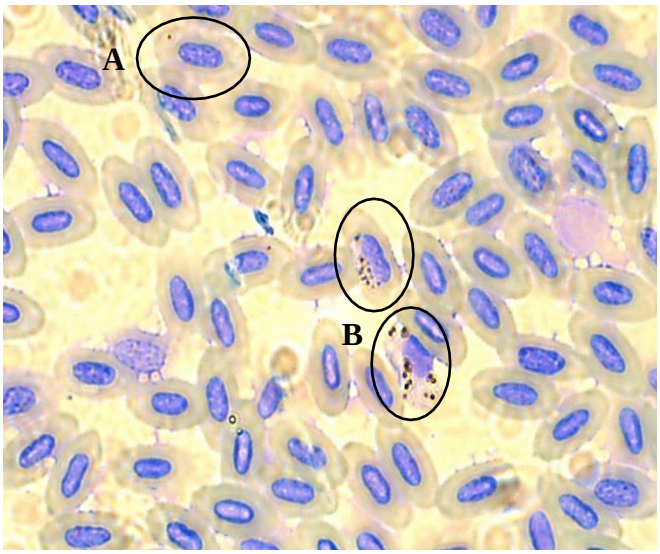

Figure 3. Bird blood smear of a *Coeligena torquata* specimen, a hummingbird also known as Collared Inca. The microscopic examination of the blood smear stained with Giemsa indicates that it is a *Plasmodium* parasite since the infection is found in the bird's red blood cells. (A) A red blood cell parasitized with a *Plasmodium* merozoite. (B) Red blood cells parasitized with *Plasmodium* schizonts. The photography was taken using the professional light optical microscope Leica model DM3000.

Differentiation of most haemosporidian parasites, except for *Plasmodium*, is commonly performed by observing morphological features of the mature gametocytes found in blood smears. Several new malaria parasite species are described every year, therefore, it is important to carry out a proper analysis of the blood samples for an accurate diagnosis. (Outlaw & Ricklefs, 2014) The possibility of providing adequate and timely treatment for malaria depends on the rapid and efficient diagnosis of the disease (Montoya et al., 2008). Among the techniques used to identify veraciously an infection by *Plasmodium* in wild birds are serological and molecular diagnostics. Furthermore, combining these methods with a parasitological analysis is a good form to obtain more accurate diagnosis results.

Parasitological Diagnosis

Avian malaria can be detected and diagnosed by microscopic examination of blood smears of birds and stained with the Giemsa reagent. Some of the advantages associated with this technique are its simplicity and low cost (Montoya et al., 2008). Moreover, this detection method has helped to reveal that the *Plasmodium relictum* species is the most common species causing avian malaria. (Valkiūnas et al., 2018) However, it is not the most reliable technique for avian malaria

identification. Hence, the use of molecular technology allows a faster and more trustworthy parasite screening (Fallon et al., 2003).

This technique consists of analyzing blood smears under the microscope and counting the parasitized red blood cells in each optical field. (Gulati, Song, Florea & Gong, 2013; Tostes et al., 2015). Each blood smear is fixed with absolute methanol and stained before the examination (Gulati et al., 2013). Then an optical field is chosen and the parasitemia percentage is obtained by dividing the parasitized red blood cells (pRBC) by the total number of red blood cells (RBC) and multiplying the result by 100% (see Equation 1; Taylor-Robinson & Phillips, 1994).

Serological Diagnosis

Serological methods recognize the antibodies in the blood of the host, wild birds in this case, in response to malaria parasites instead of recognizing the pathogens themselves. It is an effective technique for avian malaria diagnosis. Moreover, this method is effective in detecting malaria parasites even if they are in a stage in which parasitological and molecular diagnostics cannot detect them. This can be counterproductive sometimes because the serological diagnosis can also detect nonactive infections (Fallon et al., 2003). For example, the indirect immunofluorescence assay (IFA) can be employed since it uses a monoclonal antibody from a conserved antigenic protein in all species (Pace et al., 2019; Spencer Valero et al., 1998).

Molecular Diagnosis

Despite being less sensitive than a serological diagnosis, PCR can precisely detect several strains of nonhuman malaria, including the ones that cannot be easily identified by microscopic screening. This method is only valid for the detection of active infections, however, and its utility is higher when a serological analysis is also performed. PCR amplification technique allows the accurate detection of malaria blood parasites and commonly includes three main assays: two designed in the nuclear-encoded 18S small subunit (SSU), and one based on the cytochrome b gene encoded by the mitochondria. (Fallon et al., 2003) One of the applications of mitochondrial partial sequences of cytochrome b is the construction of phylogenetic trees with clearly separated clades since it has been used to efficiently distinguish between diverse lineages of *Plasmodium* parasites. Hence, it is an excellent marker for avian malaria diagnosis (Harrigan et al., 2014; Valkiūnas et al., 2018). Furthermore, there is evidence that supports that polymerase chain reaction

diagnostics enhance the detectability of chronic avian malaria infections 67% compared to microscopic analysis of blood smears (Jarvi et al., 2002).

3.2. Effects of Malaria on Wild Birds

3.2.1. Effects of Malaria on the Physiology and Behavior of Wild Birds

Malaria can manifest itself from asymptomatic to lethal in domestic, wild, and captive birds (Ilgūnas et al., 2019; Valkiūnas & Iezhova, 2018). Avian malaria has the potential to exert negative effects on birds fitness since it is frequently virulent to its hosts, and also affects their metabolism, behavior, survival rate, degradation of telomers, selection of mates, and, more alarming, avian reproductive success (Delhaye et al., 2018; Marzal, Bensch, Reviriego, Balbontin, & De Lope, 2008; Rivero & Gandon, 2018). Some of the effects on birds' physiology during the initial acute phase of the infection are anemia, lethargy, and appetite loss (Vogel, 2015). Moreover, some of the effects of *Plasmodium* include a pronounced decrease in red blood cells number, brain bleeding, and edemas. The spleen and liver are also affected by this pathogen, presenting enlargement and even necrosis in some occasions (Delhaye et al., 2018; Rivero & Gandon, 2018).

As mentioned before, the harmful effects of malaria on birds go beyond affecting their physiology, and they are more notorious when the individuals are in the acute phase of infection. In this stage, the behavior of the animals is also modified; their ability in eluding predators, their efficiency in finding food, and the capacity of defending their territory. After the initial severe acute phase, birds can undergo a low-level chronic stage. However, the effects of the first period are not well studied since acutely sick individuals are not easy to find (Asghar et al., 2015). One method usually used to ascertain the infection intensity is quantitative PCR (qPCR). This technique has allowed showing that the short-term physiological effects of avian malaria, can lead to significant long-term consequences such as the decrease in life span and reproductive output compared to those of non-infected birds (Asghar et al., 2015; Zehtindjiev et al., 2008). Interestingly, the life span shortening has been associated with the shrinking of telomeres, due to oxidative stress induced by the disease, which causes early cell death in birds (Asghar et al., 2015; Vogel, 2015).

3.2.2. Distribution and Diversity of Avian Haemosporidia

Plasmodiidae and Haemosporida parasites have been reported all around the globe except for Antarctica. Hence, these pathogens can also infect vectors in regions near to the Polar Circles, but not in them (Ilgūnas et al., 2019; Valkiūnas & Iezhova, 2018). Altogether, there are at least 55 species along with several newly discovered etiological agents (Ilgūnas et al., 2019). Despite avian malaria parasites being widely distributed across the globe, it has been shown that they have similar patterns of diversity to their hosts which tend co-vary with genera of Haemosporida. For example, *Plasmodium* parasites usually complete sporogony moderately fast in vulnerable vectors at medium to low temperatures. In general, *Haemoproteus* is more diverse than *Plasmodium* except in South America where *Plasmodium* is prevalent and widespread (Clark et al., 2014). Therefore, the global distribution of avian malaria species is related to climatic features (Doussang et al., 2019; Gil & Sedano, 2019). Additionally, another ecological parameter affecting the disease distribution is the conservation status of forests because habitat destruction, and land use can alter host-parasite interactions (Hernández-Lara, González-García, & Santiago-Alarcon, 2017).

It is well known that avian malaria parasites display a high genetic diversity, however, their geographic distribution patterns are not extensively studied yet. Avian haemosporidians' diversity and prevalence patterns differ depending on several environmental variables such as the habitat (Gil & Sedano, 2019). Finally, despite the advances made thanks to the utilization of the cytochrome b gene, such as the identification of hundreds of genetic lineages, not much is known about genetic diversity on the populations of avian malaria protozoans. The lack of information on this subject is partially caused by how difficult is to find suitable genetic markers. Hence, a trending topic concerning distribution and diversity of malaria protozoans is to study the genetic diversity and structure of the populations of *Plasmodium* parasites aiming to gain a better understanding of virulence patterns and host-parasite relationships. The available information concerning this topic suggests that geographic distribution of genetic diversity in malaria parasite populations can influence host and spatial distribution, along with diversity of avian malaria. Moreover, it can have some effects on population structure, for example, in host immune pressure and feeding preferences, migratory movements, and also in host and vector dispersal (Humphries, Stacy & Ricklefs, 2019; Valkiūnas & Iezhova, 2018).

3.2.3. Importance of the Infection in the Diversity of Wild Birds

It has been well established that avian malaria can exert several negative effects on birds' physiology, in addition, these effects can also influence the birds' biodiversity since the disease represents a threat to life span and reproductive success (Ilgūnas et al., 2019; Kimura, Darbro, & Harrington, 2010). This disease can display two types of infection in birds: blood pathology and organ damage caused by exo-erythrocytic merogony (Ilgūnas et al., 2019). Thereby, avian malaria represents a hazard of great importance to wild birds' biodiversity because it reduces lifetime reproductive success along with low life expectancy compared to uninfected birds (Asghar et al., 2015; Moens & Pérez-Tris, 2016).

An additional variable affecting the distribution of the disease, and therefore the biodiversity of wild avifauna, is the conservation status of forests because both, seasonality and habitat destruction, can alter host-parasite interactions. This means that the ecological parameters of avian malaria parasites respond to seasonality and different types of land use. Generally, a lower prevalence is expected in locations with warmer climates and well-preserved or primary forests (Asigau & Parker, 2018; Hernández-Lara et al., 2017) Finally, the avian malaria effects are especially calamitous when new parasite lineages are unintentionally introduced into a host population that was never infected before by that specific lineage (Rivero & Gandon, 2018). Conversely, birds that live in areas where malaria is endemic are not mortally affected (Rivero & Gandon, 2018).

3.2.4. Malaria Vector Control Methods

Malaria is a common vector-borne disease; therefore, vector control is a major public health responsibility since mosquito bites can cause not only malaria to humans but several other diseases (Martínez de la Puente et al., 2020; Raghavendra et al., 2011). Hence, current methods of malaria control in humans have focused on vector control techniques such as the use of transmission-blocking drugs (Primaquine) and insecticide-treated nets (ITNs; Meibalan & Marti, 2016; Spencer et al., 2016). Moreover, in the present, vector control methods mainly include chemical, biological, natural products from plants, and environmental management (Raghavendra et al., 2011).

Chemical methods are considered the most important element for vector control. Chemical control includes the application of insecticides, such as dichlorodipehnyltrichlroethane (DDT), to manage adult vector populations. This pesticide was first introduced for mosquito control in the twentieth century, and prohibited by the environmental protection agency (EPA) in 1972. The main concern regarding human health is related with the DDT residues found in several vital organs. However, this insecticide can still be used during malaria outbreaks. Additionally, pesticides can be applied by different methods such as indoor residual sprays (IRS), which consists on spraying the walls and roofs of houses to kill the vector; and space spray, which refers to the heating of pesticides to form a smoke like cloud. The latest chemical applying technique most be only used in emergencies and has to coincide with the highest activity period of mosquitoes (Raghavendra et al., 2011).

One non-chemical control method are the blocking-transmission vaccines which interrupt malaria transmission by producing antibodies against antigens during parasite sporogony, thereby preventing the infection of the insect-vector. The vertebrate host will produce antibodies against the gametocytes to block their fertilization, and against the oocysts to block their mobilization into the vector's midgut. However, these vaccines need further improvement to function optimally (Spencer et al., 2016; Spencer Valero et al., 1998). Additionally, several control methods have been proposed recently, to protect the avifauna in their habitat. Nevertheless, these options are not logistically, economically, nor ecologically feasible yet (Fortini, Kaiser, & LaPointe, 2020).

3.3. Experimental infections

3.3.1. Experimental infections to analyze the effect on the physiology of birds

In 1898, Sir Ronald Ross used avian malaria to prove that the parasite transmission was accomplished by mosquitos (Cox, 2010; Huff, 1965; Raghavendra et al., 2011; Rivero & Gandon, 2018). Ronald accomplished this discovery, along with the explanation of the pathogen life cycle within the mosquito, by working with laboratory-bred *Culex* mosquitoes that were fed with *Plasmodium* infected and uninfected birds' blood (Rivero & Gandon, 2018). Then, many of studies were performed during the first half of the 20th-century, not only concerning the transmission, life cycle, and the effects on the avian fauna but also regarding drugs for the treatment of human malaria. Nevertheless, experimental research on avian malaria greatly declined after the discovery

of rodent malaria in 1948, and the thriving experimental approach of infecting monkeys with human malaria in 1966. Nonetheless, bird species still represent a successful model to study human malaria because the species of *Plasmodium* infecting both are closely related phylogenetically, including the most virulent species to humans, *P. falciparum* (Palinauskas, Valkiūnas, Bolshakov & Bensch, 2008). In view of these advantages, experimental studies are useful to gain a better understanding of how to treat the disease and its possible effects on birds' physiology and biodiversity (Ilgūnas et al., 2019).

Experimental infections are usually performed in a bird species of interest; the individuals are infected with an isolated parasite which is commonly *P. relictum* (Jarvi et al., 2002). Thereby, several experimental studies have been carried out all over the world; Hawaii being one of the most studied locations since avian malaria is associated with biodiversity loss of many Hawaiian endemic bird species. (Atkinson, Dusek, Woods, & Iko, 2000; Fortini et al., 2020; Feldman, Freed, & Cann, 1995; Liao, Atkinson, LaPointe, & Samuel, 2017; Samuel, Liao, Atkinson, & LaPointe, 2020; Whiteman et al., 2005).

One of the described studies was realized by Atkinson et al. (2000), on the Hawaii Amakihi, *Hemignathus virens*, that was experimentally-infected to measure morbidity and mortality. Thirty-two specimens were captured in 1993 from a dry and cold habitat. The results obtained agree with other experimental studies previously done on native Hawaiian forest species. Laboratory-infected birds showed a decrease in weight due to a decline in feeding during the first 13 days after infection. The authors showed that this species was more physiologically capable to survive than other experimentally infected species such as Iiwi, *Vestiaria coccinea*, but had similar mortality than the Apapane, *Himatione anguinea*, which was captured in similar habitats. Thus, they demonstrated that the physiological capacity to survive, of the Hawaii Amakihi and the Apapane, was associated with their geographic distribution and altitudinal range. Additionally, the survival capacity is correlated to the diversity of major histocompatibility complex (Mhc) since they found lower Mhc diversity in *V. coccinea* than *H. virens* (Atkinson et al., 2000). Then, the capacity to overcome the infection by avian malaria parasites is associated to Mhc diversity since a higher diversity means a higher chance for birds' immune system "to recognize and process specific malarial-encoded peptides" (Atkinson et al., 2000, p. 202). Finally, the authors suggest that other immunogenetic factors, such as several loci and other genetic systems, may also be involved in the recovery capacity of birds from malarial infections (Atkinson et al., 2000).

Lastly, it is important to mention that studies using experimentally infected birds have allowed researchers to learn much about the effects of avian malaria in birds' physiology. For example, Ilgūnas et al. (2019) demonstrated that avian malaria protozoans not only infect blood but also are found in several internal organs like the heart, liver, brain, lungs, spleen, and kidneys, causing tissue damage. These researchers infected passerine birds at the Biological Station of the Zoological Institute of the Russian Academy of Sciences on the Curonian Spit in the Baltic Sea. Their study also permitted them to conclude that one of the primary causes of mortality of sick birds is cerebral ischemia and ensuing brain damage. All these discoveries represent a step closer to the development of new treatments for avian malaria, not only for the erythrocytic phase of the infections but also for the tissue stage which causes organ failure in birds (Ilgūnas et al., 2019).

4. AVIAN MALARIA IN PREMONTANE FORESTS OF WESTERN ECUADOR

4.1. Methodology

4.1.1. Reagents

First, slide blood smears are fixed with the absolute methanol, 99.8%. Then, the Giemsa reagent is used for the visualization of birds' red blood cells since it stains the DNA present in the nucleated erythrocytes. After the samples are stained, they are washed with distilled water and air-dried, to later be observed with immersion oil by a microscope from the 100X objective.

4.1.2. Equipment

Levels of parasitemia were determined using a professional light optical microscope Leica model DM3000, and optical microscope Leica model DM300 to take the pictures.

4.1.3. Sampling material

The sampling material employed for this study included mist-nets for bird capture; portable scale and bird measuring tools, such as a Venier instrument, to measure and weigh the individuals; gloves, lancets, cotton, and 1 ml/cc sterile syringes (VANJERIN) for blood sampling; microscope slides (CITOPLUS) and coverslips (Knittel GLASS) to perform the blood smears; filter paper to sample a blood drop for PCR; adhesive tape and permanent marker to label the samples; and finally, paper towels for transporting the microscope slides with the smeared blood.

4.1.4. Sampling areas

The sampling of birds was performed in two different reserves managed by the Mindo Cloudforest Foundation (MCF) in Pichincha province: the Milpe Bird Sanctuary located in San Miguel de Los Bancos (0°02'12.9''N 78°52'12.8'' W, 1150 m.a.s.l.), and the Hacienda Puyucunapi located near Nanegalito (0°01'33.5''N 78°41'48.5'' W, 2000 m.a.s.l.). The coordinates were determined on Google Maps (July 10, 2020). Both study sites are part of a 1000 m elevational gradient. They have a similarly humid climate, Puyucunapi being colder than Milpe, with an annual average relative humidity of 80%, and average annual cumulative rainfall of approximately 2525 mm across both areas. (Anthelme, Lincango, Gully, Duarte, & Montúfar, 2011; Córdova et al., 2016; Guerra-Correa, Merino-Viteri, Andrango, & Torres-Carvajal, 2020; Lastra, Yánez, Garzón, & Salcedo, 2020; Rodríguez, Castro, Marín, Roldán, & Viteri, 2019). Moreover, there are permanent stream and stagnating water sources (collecting in leaves and bromeliads, for instance) at both locations (Caisatoa, 2016). In the Milpe area, 14 birds were captured during a one-day field trip, while at Puyucunapi 50 individuals were captured in two different field trips.

4.1.5. Bird capture and blood sampling process

For this study, the birds were captured by using mist-nets. All captured birds belonged to the family Trochilidae (hummingbirds) and the order Passeriformes (songbirds; see Appendix 7). Moreover, birds were marked or distinguishing characteristics were recorded such that recaptures were not resampled. The sampling method included blood smears and collecting a blood sample on filter paper for molecular diagnosis with PCR (see Future Work). First, each bird was weighed (gr) and measured (mm). The measures taken were exposed culmen, tarsometatarsus, tail, and wing length (Baldwin, Oberholser & Worley, 1931). Other data collected included the presence and state of brood patch, condition of the cloaca, fat accumulation, muscle volume, and molting presence (Ralph, Martin, Geupel, DeSante & Pyle, 1993). The color of the ring was also registered for passerine birds, and the ring was always put on the left leg. Blood smears were obtained by taking a drop of blood from the metatarsal vein in small birds, such as hummingbirds, or the brachial vein in passerines. The drop was placed on the slide and the blood smear was performed immediately by placing a spreader slide (coverslip) at an angle of 45° allowing blood to be adsorbed by the slide and then drawing it away from the blood drop to spread it on the glass. A duplicate was made if enough blood was available. Once the birds were identified, weighed,

measured, and the blood sample was taken, birds were checked to make sure bleeding had stopped and released. In the case of hummingbirds, they were usually fed with sugar water before being freed. Passerines were often given water to drink. After the blood smears were air-dried, they were fixed with absolute methanol for 1 to 3 minutes and wrapped in paper towels for transport. Samples were stained with the Giemsa reagent in the laboratory at Yachay Tech University (Richard et al., 2002; De La Torre et al., 2020).

4.1.5. Specimen staining and parasitological diagnosis

The fixed blood smears were stained using the Giemsa's reagent diluted 1:5 with phosphate-buffered saline (PBS). Then, each smear was covered with the Giemsa solution for 10 minutes. After that, they were delicately washed with tap water and placed vertically to air dry (Iezhova, Valkiūnas, & Bairlein, 2005; Sullivan et al., 2005; De La Torre et al., 2020). Once dry, the stained samples were observed under the optical Leica DM300 microscope for parasitological diagnosis. The samples were observed with an amplification of 100X using immersion oil. An optical quadrant was chosen and the parasitemia percentage was obtained by counting the total number of blood cells and the parasitized red blood cells. The following formula was employed:

$$pRBC\ \% = \frac{No.\ pRBC}{No.\ RBC}\ x\ 100\% \qquad \text{(Equation 1)}$$

where pRBC stands for parasitized Red Blood Cells and RBC means Red Blood Cells (Matta & Rodríguez, 2001; Taylor-Robinson & Phillips, 1994; Valkiūnas & Iezhova, 2018). An average of 200 erythrocytes was counted per field of view, and approximately 10 fields were examined per slide. The best blood smears were photographed by using the professional light optical Leica model DM3000 microscope (Figure 2). Additionally, the prevalence at each location was calculated applying the following formula, which expresses *Plasmodium* abundance (see Figures 5 and 6; Galen & Witt, 2014):

$$Parasitemia\ prevalence = \frac{No.\ of\ infected\ hosts}{Total\ No.\ of\ screened\ hosts}\ x\ 100\% \qquad \text{(Equation 2)}$$

The combined infection rate was obtained by dividing the total number of infected individuals by the total number of sampled specimens (Figure 7; Galen & Witt, 2014).

4.1.6. Statistical analysis

From the 50 birds captured at Puyucunapi Pilot Project, 47 were used for the statistical analysis since one of them did not have the corresponding blood smear while for the other two it was impossible to determine he parasitemia due to poor quality of smears (see Appendix 1). On the other hand, of the 14 birds captured in the Milpe Bird Sanctuary, 13 samples were used for the statistical section of this study because it was not possible to do a blood smear of one of the specimens (see Appendix 2). Hence, the statistical analysis concerning elevation was performed for a total of 60 samples presented as parasitized red blood cells' percentages on average parasitemia.

First, a Wilk-Shapiro test was carried out to determine if the parasitemia data had a normal distribution (see Appendix 5). Since normality was not supported, a nonparametric Mann-Whitney U test was used to examine independent samples. This test corresponds to the Student's t-test used for parametric samples (see Appendix 5). Both tests can be considered analogous since their main objective is to confirm or deny the existence of statistically significant differences between the two groups to be studied (MacFarland & Yates, 2016). Moreover, Mann-Whitney U test was performed to test if the birds' sex is not related to the degree of malaria infection (see Appendix 6). For this analysis, 37 individuals were taken into account because for the other 23 sex could not be determined. Among the categorized individuals, 19 were females and 18 were males. R-Studio (2013) version 3.0.1 was used for all statistical analyses.

4.2. Results and Discussion

4.2.1. Results

A total of 64 birds of 21 species were captured at the two localities. From these, 50 were captured at the Puyucunapi (Nanegalito), and 14 at the Milpe Bird Sanctuary (Milpe). Four individuals were discarded from the total due to two possible reasons: lack of blood smear of the specimen, or poorly performed smear which impeded the parasitemia percentage to be calculated (see Appendices 1, 2). Thereby, the sample sizes used for the statistical analysis were 60 (N=60), 47 for Puyucunapi, (Table 1), and 13 from Milpe (Table 2). Of the two studied areas, Puyucunapi was the one with greater α-diversity with a total of 14 different bird species (Table 1), while in the Milpe area seven different bird species were captured (Table 2).

Table 1. Mean parasitemia percentages per species of the Nanegalito area (Puyucunapi Pilot Project at MCF).

Number	Species	Common name	Number of individuals	Sex	Mean Parasitemia (%)
1	*Adelomyia melanogenys*	Speckled Hummingbird	1	Unk	5%
2	*Aglaiocercus coelestis*	Violet-tailed Sylph	7	2F, 5M	6%
3	*Boissonneaua flavescens*	Buff-tailed coronet	4	4 Unk	5%
4	*Coeligena torquata*	Collared Inca	1	M	4%
5	*Coeligena wilsoni*	Brown Inca	11	1F, 10 Unk	6%
6	*Dendrocincla tyrannina*	Tyrannine Woodcreeper	1	Unk	2%
7	*Heliodoxa imperatrix*	Empress Brilliant	1	F	9%
8	*Heliodoxa rubinoides*	Fawn-breasted Brilliant	13	9F, 4M	4%
9	*Mionectes striaticollis*	Streak-necked Flycatcher	1	Unk	6%
10	*Myiophobus fasciatus*	Bran-colored Flycatcher	1	Unk	6%
11	*Ocreatus underwoodii*	White-booted Racket-tail	1	F	1%
12	*Pachyramphus versicolor*	Barred Becard	1	F	9%
13	*Phaethornis syrmatophorus*	Tawny-bellied Hermit	2	Unk	2%
14	*Urosticte benjamini*	Purple-bibbed Whitetip	2	1F, 1M	5%

F=Female, M=Male, and Unk= Unknown

Table 2. Mean parasitemia percentages per species of the Milpe area (Milpe Bird Sanctuary at MCF).

Number	Species	Common name	Number of individuals	Sex	Mean Parasitemia (%)
1	*Dendrocincla fuliginosa*	Plain-brown Woodcreeper	1	Unk	5%
2	*Florisuga mellivora*	White-necked Jacobin	1	F	IN
3	*Glyphorynchus spirurus*	Wedge-billed Woodcreeper	1	Unk	3%
4	*Heliodoxa jacula*	Green-crowned Brilliant	4	1F, 3M	5%
5	*Machaeropterus deliciosus*	Club-winged Manakin	1	M	4%
6	*Premnoplex brunnescens*	Spotted Barbtail	1	M	4%
7	*Thalurania fannyi*	Green-crowned Woodnymph	5	2F, 2M, 1 Unk	3%

F=Female, M=Male, and Unk= Unknown

Both localities had different species, i.e., no species was repeated between the two sampling areas (Figure 4). At Puyucunapi, 45 of the 47 individuals were infected representing 96% of the sample (Figure 5). The two individuals that did not present any signs of malaria infection were two females of *Heliodoxa rubinoides* species. Moreover, the highest level of parasitemia was 9% found in two species, *Heliodoxa imperatrix* and *Pachyramphus versicolor* (Figure 4). On the other hand, at Milpe all 13 birds were infected, i.e., a prevalence of 100% (Figure 6). The highest parasitemia level at this location was 5% corresponding to *Dendrocincla fuliginosa* and *Heliodoxa jacula* (Figure 4).

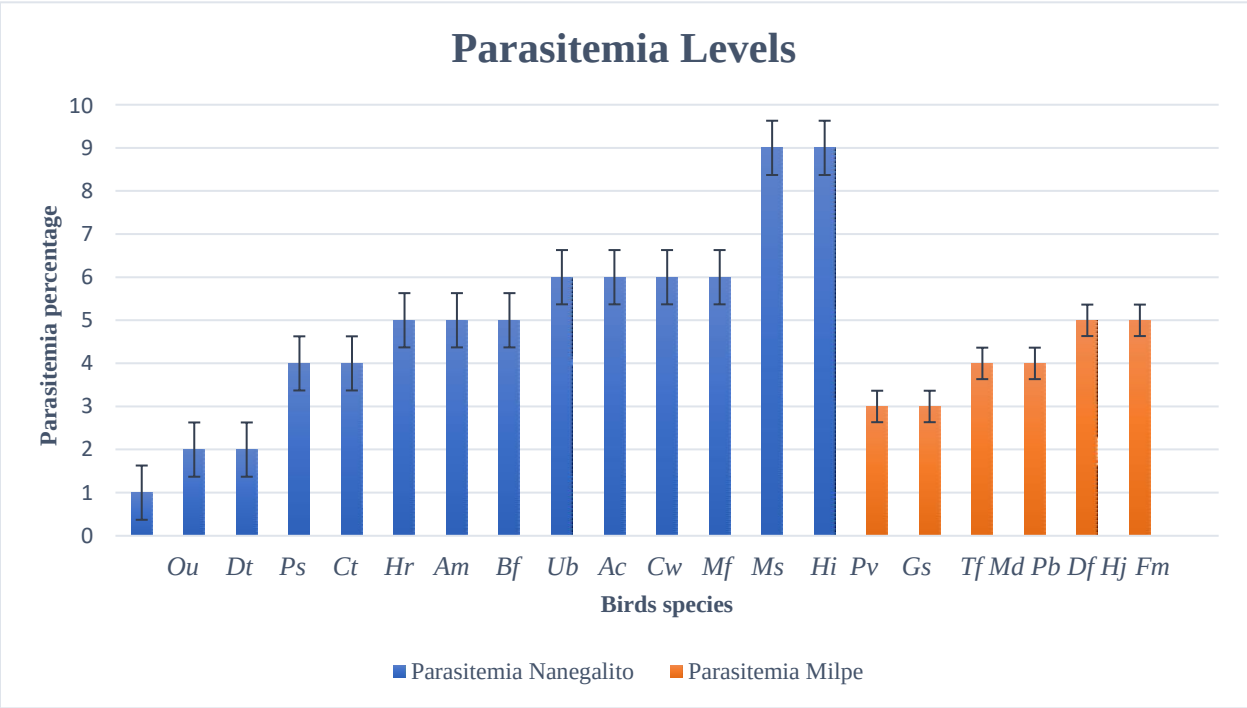

Figure 4. Parasitemia levels in the different bird species captured at the two sampling areas, Puyucunapi Pilot Project at Nanegalito (blue bars), and Milpe Bird Sanctuary at Milpe (orange bars). The abbreviations for the birds' species names are *Ocreatus underwoodii* (*Ou*), *Dendrocincla tyrannina* (*Dt*), *Phaethornis syrmatophorus* (*Ps*), *Coeligena torquata* (*Ct*), *Heliodoxa rubinoides* (*Hr*), *Adelomyia melanogenys* (*Am*), *Boissonneaua flavescens* (*Bf*), *Urosticte benjamini* (*Ub*), *Aglaiocercus coelestis* (*Ac*), *Coeligena wilsoni* (*Cw*), *Myiophobus fasciatus* (*Mf*), *Mionectes striaticollis* (*Ms*), *Heliodoxa imperatrix* (*Hi*), *Pachyramphus versicolor* (*Pv*), *Glyphorynchus spirurus* (*Gs*), *Thalurania fannyi* (*Tf*), *Machaeropterus deliciosus* (*Md*), *Premnoplex brunnescens* (*Pb*), *Dendrocincla fuliginosa* (*Df*), *Heliodoxa jacula* (*Hj*), *Florisuga mellivora* (*Fm*).

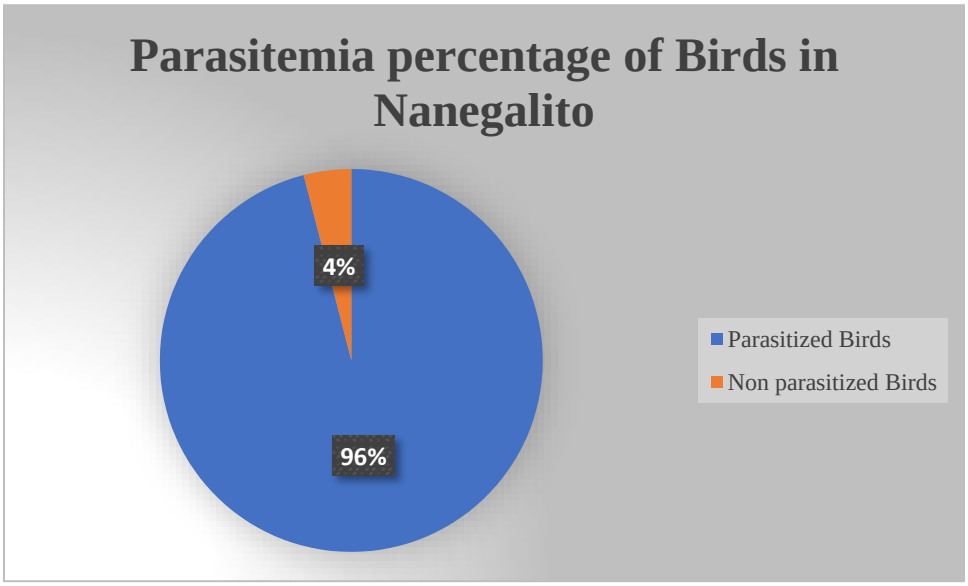

Figure 5. *Plasmodium* prevalence at Puyucunapi Pilot Project (Nanegalito).

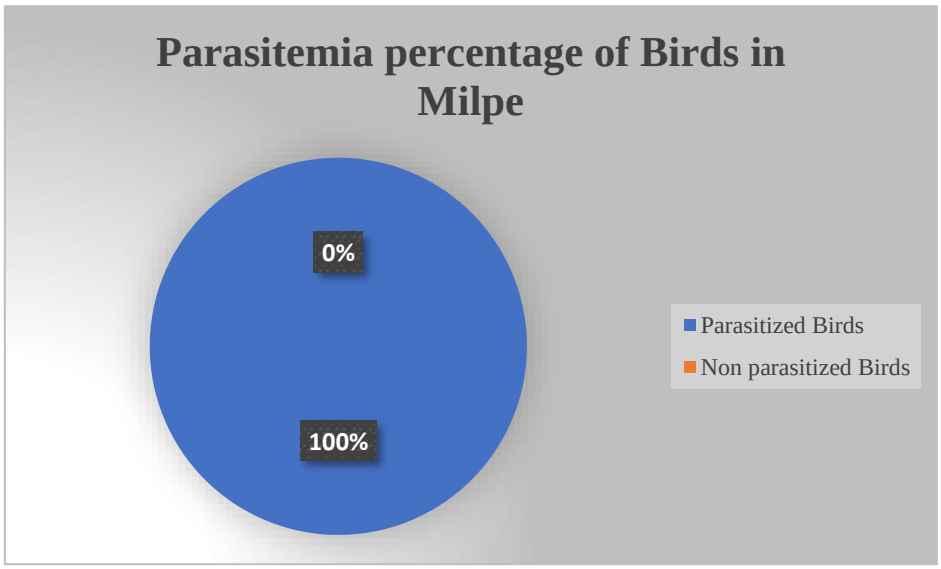

Figure 6. *Plasmodium* prevalence at Milpe Bird Sanctuary (Milpe).

Additionally, the combined infection rate of the studied sites is 97% with 58 of 60 individuals infected (Figure 7). Finally, the difference between the mean parasitemia percentage of both localities is very small since Milpe has an average parasitemia of 4% and Nanegalito of 5% (Figure 8).

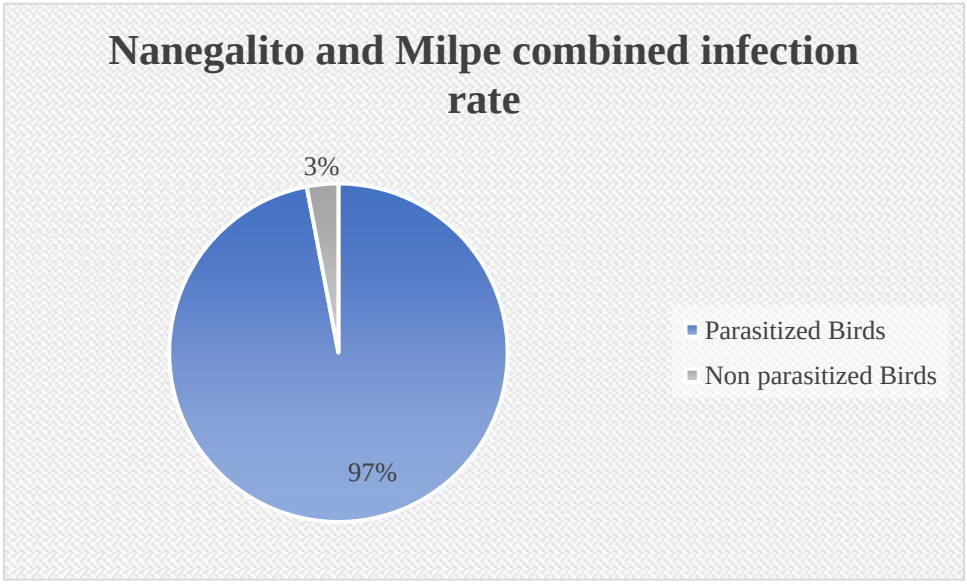

Figure 7. Total parasitemia representation of both humid forest locations, Nanegalito and Milpe.

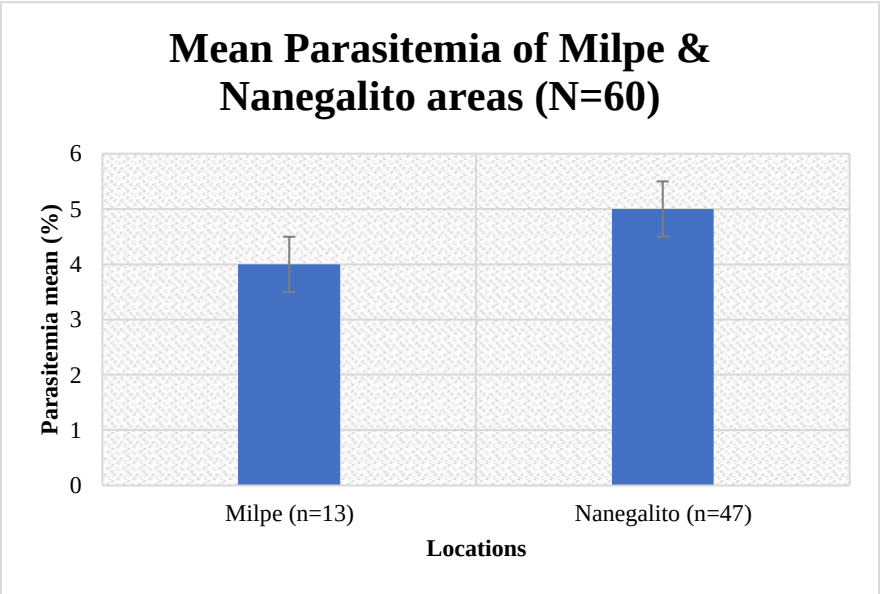

Figure 8. Milpe and Nanegalito mean parasitemia percentages.

A statistical analysis was carried out to determine if there was a statistically significant difference between the data of the two studied areas concerning the altitudinal parameter. Therefore, by performing a Wilk-Shapiro test in R-Studio, it was demonstrated that the data is not normally distributed (see Appendices 3, 4, 5). The p-value obtained was 0.01504 which indicates that there is evidence to reject the normality assumption since the p-value is less than $\alpha = 0.05$ (see Appendix 5; Henderson, 2006). Additionally, a histogram of each site was done to visually show that neither of them follows a normal distribution (see Appendix 3). A boxplot along with a histogram of both locations was also performed to show that the collected data is not normally distributed (see Appendix 4).

Given that the data does not follow a normal distribution, a Mann-Whitney U test was run to define if the null hypothesis (H0) can be rejected.

- **H_0:** There is no statistically significant difference between the mean parasitemia percentages of Nanegalito and Milpe areas concerning the altitudinal parameter.
- **H_a:** There is a statistically significant difference between the mean parasitemia percentages of Nanegalito and Milpe areas concerning the altitudinal parameter.

The obtained p-value was 0.3181 which means there is no evidence to reject the null hypothesis because the p-value is greater than $\alpha = 0.05$. (see Appendix 5; MacFarland & Yates, 2016). Hence,

by running this test it is suggested that a difference of almost a thousand meters between the two localities studied has not resulted in a statistically significant difference in their mean parasitemia percentages. On the other hand, another Mann-Whitney U test was conducted to analyze if the sex of the individuals influences the degree of infection (see Appendix 6). The resulting p-value was 0.1122, and since is not less than alpha it is suggested that the birds' sex does not influence the avian malaria parasitemia percentage.

4.2.2. Discussion

Prevalence and parasitemia

The prevalence of *Plasmodium* between the two studied areas, Nanegalito and Milpe, was proportional to what was expected for the humid forest and the presence of water bodies (Anthelme et al., 2011; Córdova et al., 2016; Guerra-Correa et al., 2020; Lastra et al., 2020; Rodríguez et al., 2019), being 96% (Figure 5) and 100% (Figure 6), respectively. Hence, the prevalence obtained agrees with the hypothesis of a higher abundance at a lower altitude, albeit only slightly. However, by performing the Mann-Whitney U test it was demonstrated that there is not enough evidence to claim that the elevation difference is the parameter responsible for the presented outcome (MacFarland & Yates, 2016). The results are consistent with the literature since there is evidence that supports that "avian haemosporidians extend upslope to the limit of available bird habitat" (Galen & Witt, 2014, p. 10). In other words, a higher elevation does not imply a lower prevalence, and there is evidence that the opposite is occurring (Harrigan et al., 2014).

Additionally, by comparing the results from this work to the data provided by Dr. Spencer's research group in the inter-Andean dry forest of Imbabura Province (data not published), it was found that maximum parasitemia levels were similar with that of 9% in northwestern Pichincha and 8% in the warmer and less elevated site studied in the tropical dry forest. Prevalence depended to a small degree on elevation. On average, the humid forest has a total prevalence of 97% while the dry forest of 80%, showing that elevation along with several eco-geographical features influence the infection degree in birds since they affect the proliferation of the insect-vector.

Moreover, this study reports a total of 21 bird species that inhabit both humid forest localities, while in the dry forest study a total of 18 bird species were sampled in the three studied areas. From the 21 species captured in the humid forest, seven species were from the Milpe area, which

represents the less elevated of the two examined sites, while the other 14 belonged to the Nanegalito area. In total, 20 bird species were used for the analysis. Except for *Myiophobus fasciatus* there is no overlap between the species caught in the humid and the dry forests. Incidentally, in the dry forest *M. fasciatus* was one of the most parasitized species with a parasitemia percentage of 6%. This species serves as an example of the complex influence of altitude and eco-geographical features on the infection degree in birds. A 2.5% mean parasitemia percentage in Las Yunguillas (dry forest) at an average altitude of 2050 m.a.s.l., 6% in Nanegalito at 2000 m.a.s.l., and 8% in Salinas at 1580 m.a.s.l., suggest that altitude may interact with other factors in determining parasitemia percentage. Furthermore, the humid forest presented a total prevalence of 97% while the dry forest of a total prevalence of 80%. This suggests that elevation along with several eco-geographical features slightly influence the infection degree in birds since they affect the proliferation of the insect-vector (personal communication from Professor Lilian Spencer, Ph.D.).

Effects on wild birds

There are few studies concerning avian haemosporidians that cause malaria in Latin America, Ecuador included, with some reports from the Galapagos Islands. Therefore, the humid forest of Pichincha Province is a location of interest (Asigau & Parker, 2018; Palmer et al., 2013; Whiteman et al., 2005). Avian malaria ranges from asymptomatic to lethal in domestic, wild, and captive birds (Ilgūnas et al., 2019; Valkiūnas & Iezhova, 2018). Because it is frequently virulent to the hosts (Marzal et al., 2008; Rivero & Gandon, 2018), it has the potential to cause harmful effects on birds' fitness Some of the effects on birds' physiology during the initial acute phase of the infection are anemia, lethargy, and appetite loss (Vogel, 2015). Moreover, *Plasmodium* can also provoke a pronounced decrease in red blood cell number, brain bleeding, and edemas. The spleen and liver are also affected by this pathogen, presenting enlargement and even necrosis in some occasions. Nonetheless, birds that live in areas where malaria is endemic are usually not mortally affected; arguably this is the case at both studied humid forest locations of Pichincha Province (Delhaye et al., 2018; Rivero & Gandon, 2018). If the trends observed in this study hold up with greater sample sizes, *Heliodoxa imperatrix* and *Pachyramphus versicolor* might be at risk of extirpation as they presented the highest degrees of parasitemia. Conversely, the species at lower risk would be *Ocreatus underwoodii* with 1% of parasitemia.

5. FUTURE WORK

5.1. PCR Detection of *Plasmodium* parasites

Microscopic or parasitological diagnosis is a simple and economical option for *Plasmodium* parasites detection since it needs a few materials and equipment to be performed (Montoya et al., 2008). However, for most effective differentiation of these hematozoan parasites, molecular methods are employed (Martínez et al., 2009). Molecular detection by sensitive PCR was intended for this study, in addition to microscopic detection, but it was not finished due to the sanitary emergency related to COVID-19. The purpose of this section is to show how I would have conducted experiments and the standardization procedure that needs to be performed to establish the optimal parameters and reagents to be used for the detection of the parasite in the 60 sampled specimens.

The first steps of the standardization stage were achieved before the health emergency. The blood was extracted from five different chickens and one peacock in Santiago del Rey in Imbabura province. Blood samples collected into BD Vacutainer EDTA K2 blood collection tubes. Only three blood samples were stored correctly in the blood collection tubes; as a result, only those three samples, along with a peafowl sample, were used for DNA extraction. Moreover, human blood was used as a control since the patient could not have been infected with avian malaria. The presence of malaria infection in the domestic birds used in this process was first determined by parasitological diagnosis following the same dying and counting procedure as that employed for wild birds.

DNA extraction was done following the standard steps of the Qiagen DNeasy Blood and Tissue Kit for nucleated blood (birds) and nonnucleated blood (human control; Dadam et al., 2019). The DNA was then amplified by PCR in the Thermo Scientific thermocycler using the following parameters: 94 °C during 10 minutes for the polymerase activation, 40 cycles at 95 °C for 40 seconds, 60 °C for 1 minute for the suggested primers for *Plasmodium* detection (58 °C for *Haemoproteus*), 72 °C for 1 minute, and 72 °C for 10 minutes for the final extension (see Appendix 8A; Martínez et al., 2009). The final PCR mixes had a volume of 25 µL when placed in the thermocycler. The fifth standardization process trial finally gave the expected results (see Appendix 8B).

5.2. Future work and suggested primers

Venous blood of the 60 individuals was collected in filter paper for the molecular detection of *Plasmodium*. Two DNA extraction procedures that can be performed for this sampling technique are 1) obtaining DNA by the washing method, and 2) DNA extraction by the Saponin/Chelex-100 method (Rachid et al., 2010). The primers employed for the standardization procedure and intended to be used for future molecular detection of *Plasmodium* are Plas-F (5'-GTA ACA GCT TTT ATG GGT TAC-3') and 4292Rw (5'-TGG AAC AAT ATG TAR AGG AGT-3') with a length of 422 bp. (Martínez et al., 2009) Both primers were obtained from Invitrogen (ThermoFisher Scientific Corporation). After the amplification, all the products of the PCR must be run in a 1.5% agarose gel, with a dye loading buffer to visualize the amplified fragment, and a DNA ladder to verify if the individual sample is infected with *Plasmodium*. Finally, each gel needs to be visualized and photographed under ultraviolet light (Tostes et al., 2015). Unfortunately, the work of *Plasmodium* determination by PCR could not be finished, due to the current situation of the pandemic but will be continued in the future.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

Malaria is a tropical zoonotic disease that affects several vertebrate classes like mammals, reptiles, and birds. Furthermore, the infection is specific for each of them, i.e., the protozoan species infecting birds do not infect mammals or reptiles, and vice versa. This disease is caused by haemosporidians or protozoans of the Haemosporida order, of phylum Apicomplexa. Two genera of this order constitute the etiological agents for avian malaria: *Plasmodium*, and *Haemoproteus*. They can be often confused since they are paraphyletic and share several physiological similarities such as the appearance of their gametocytes. Consequently, it is necessary to use more than one diagnosis technique to distinguish between both genera. Avian malaria parasites are distributed worldwide while the *Plasmodium* genus is especially concentrated in South America. Hence, it is important to know the different effects of the infection on birds' health and biodiversity.

The statistical analysis performed showed that there is not enough evidence to claim that the altitude parameter is directly related to avian malaria incidence in the current study. This result can be due to the similar eco-geographical features both locations exhibit, or due to the relatively

low sample size of the study. Moreover, it was suggested that the sex of bird species does not affect the infection degree of avian malaria as indicated in the literature. Finally, the localities studied in the humid forest are very similar ecologically; hence, a significant difference in avian infection was not observed. Additionally, from the 20 bird species analyzed in this study, *Heliodoxa imperatrix* and *Pachyramphus versicolor* are the species with the most elevated potential risk of extirpation because they have the highest degree of parasitemia.

Recommendations

- To let the Giemsa reagent act on the blood smears until they are completely stained, i.e., for 10 minutes. This is important because if they are not properly stained, the malaria hematozoan will be difficult to identify during the parasitological diagnosis. On the other hand, if the smears are over-stained, the sample will be very dark and, therefore, indistinguishable under the microscope. In summary, it is fundamental to always standardize the required staining time for the Giemsa reagent.
- To have a higher sample size since small sample sizes can contribute to decreasing the significance level of the findings, in the case of this study of the Mann-Whitney U test performed on elevation (Atkinson et al., 2000). However, the sample size could not be increased due to the sanitary emergency.
- Further application of another diagnosis technique, besides the parasitological diagnosis, to verify the *Plasmodium* infection in the sampled birds. For example, the use of indirect immunofluorescence assay (IFA) using a monoclonal antibody from a conserved antigenic protein in all species (Pace et al., 2019; Spencer Valero et al., 1998; Valkiūnas et al., 2018).

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8. APPENDICES

Appendix 1.

Puyucunapi Pilot Project Sampling Data. A total of 50 birds of 14 species were captured, from that number, three were excluded from the statistical analysis as showed in this table. The one marked as NA and colored in red was not included because there was no blood smear of that individual while the two marked as IN and colored in orange were left out because the smears were poorly done and, hence, the parasitemia percentage could not be determined.

NANEGALITO (OCTOBER 27th AND 28th, 2018)						
Individual #	**Sample #**	**Common name**	**Species**	**Code**	**Parasitemia (%)**	**Parasitemia Mean (%)**
1	**1**	Brown Inca	*Coeligena wilsoni*	S-27/10/2018-Coewil-1A	2%	9%
	2	Brown Inca	*Coeligena wilsoni*	S-27/10/2018-Coewil-1B	16%	
2	**3**	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-27/10/2018-Helrub-2A	4%	6%
	4	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-27/10/2018-Helrub-2B	7%	
3	**5**	Brown Inca	*Coeligena wilsoni*	S-27/10/2018-Coewil-3A	2%	4%
	6	Brown Inca	*Coeligena wilsoni*	S-27/10/2018-Coewil-3B	6%	
4	**7**	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-27/10/2018-Helrub-4A	3%	5%
	8	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-27/10/2018-Helrub-4B	7%	
5	**9**	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-27/10/2018-Helrub-5A	4%	4%
	10	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-27/10/2018-Helrub-5B	3%	
6	**11**	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-28/10/2018-Helrub-6A	4%	7%
	12	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-28/10/2018-Helrub-6B	10%	
7	**13**	Bran-colored Flycatcher	*Myiophobus fasciatus*	S-28/10/2018-Myifas-7A	8%	6%
	14	Bran-colored Flycatcher	*Myiophobus fasciatus*	S-28/10/2018-Myifas-7B	4%	

8	**15**	Violet-tailed Sylph	*Aglaiocercus coelestis*	S-28/10/2018-Aglcoe-8A	3%	8%
	16	Violet-tailed Sylph	*Aglaiocercus coelestis*	S-28/10/2018-Aglcoe-8B	12%	
9	**17**	Brown Inca	*Coeligena wilsoni*	S-28/10/2018-Coewil-9A	6%	7%
	18	Brown Inca	*Coeligena wilsoni*	S-28/10/2018-Coewil-9B	7%	
10	**19**	Collared Inca	*Coeligena torquata*	S-28/10/2018-Coetor-10A	5%	4%
	20	Collared Inca	*Coeligena torquata*	S-28/10/2018-Coetor-10B	3%	
11	**21**	Barred Becard	*Pachyramphus versicolor*	S-28/10/2018-Pacver-11A	8%	9%
	22	Barred Becard	*Pachyramphus versicolor*	S-28/10/2018-Pacver-11B	10%	
12	**23**	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-28/10/2018-Helrub-12A	2%	3%
	24	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-28/10/2018-Helrub-12B	3%	
13	**25**	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-28/10/2018-Helrub-13A	5%	4%
	26	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-28/10/2018-Helrub-13B	3%	
14	**27**	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-28/10/2018-Helrub-14A	6%	5%
	28	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-28/10/2018-Helrub-14B	3%	
15	**29**	Streak-necked Flycatcher	*Mionectes striaticollis*	S-28/10/2018-Miostr-15A	4%	6%
	30	Streak-necked Flycatcher	*Mionectes striaticollis*	S-28/10/2018-Miostr-15B	8%	
16	**31**	Brown Inca	*Coeligena wilsoni*	S-28/10/2018-Coewil-16A	8%	9%
	32	Brown Inca	*Coeligena wilsoni*	S-28/10/2018-Coewil-16B	10%	
NANEGALITO (FEBRUARY 9th AND 10th, 2020)						
17	**20**	Brown Inca	*Coeligena wilsoni*	S-09/02/2020-Coewil-15A	4%	4%
	21	Brown Inca	*Coeligena wilsoni*	S-09/02/2020-Coewil-15B	4%	

	22	Brown Inca	*Coeligena wilsoni*	S-09/02/2020-Coewil-16A	NA	NA
18	**23**	Violet-tailed Sylph	*Aglaiocercus coelestis*	S-09/02/2020-Aglcoe-17A	5%	5%
	24	Violet-tailed Sylph	*Aglaiocercus coelestis*	S-09/02/2020-Aglcoe-17B	4%	
19	**25**	Violet-tailed Sylph	*Aglaiocercus coelestis*	S-09/02/2020-Aglcoe-18A	4%	3%
	26	Violet-tailed Sylph	*Aglaiocercus coelestis*	S-09/02/2020-Aglcoe-18B	2%	
20	**27**	Brown Inca	*Coeligena wilsoni*	S-09/02/2020-Coewil-19A	3%	3%
	28	Brown Inca	*Coeligena wilsoni*	S-09/02/2020-Coewil-19B	3%	
21	**29**	Empress Brilliant	*Heliodoxa imperatrix*	S-09/02/2020-Helimp-20A	9%	9%
	30	Empress Brilliant	*Heliodoxa imperatrix*	S-09/02/2020-Helimp-20B	8%	
22	**31**	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-09/02/2020-Helrub-21A	3%	3%
	32	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-09/02/2020-Helrub-21B	2%	
23	**33**	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-09/02/2020-Helrub-22A	IN	3%
	34	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-09/02/2020-Helrub-22B	3%	
24	**35**	Violet-tailed Sylph	*Aglaiocercus coelestis*	S-09/02/2020-Aglcoe-23A	4%	4%
25	**36**	Tawny-bellied Hermit	*Phaethornis syrmatophorus*	S-09/02/2020-Phasyr-24A	2%	2%
	37	Tawny-bellied Hermit	*Phaethornis syrmatophorus*	S-09/02/2020-Phasyr-24B	2%	
26	**38**	Tawny-bellied Hermit	*Phaethornis syrmatophorus*	S-09/02/2020-Phasyr-25A	2%	2%
	39	Tawny-bellied Hermit	*Phaethornis syrmatophorus*	S-09/02/2020-Phasyr-25B	2%	
27	**40**	Brown Inca	*Coeligena wilsoni*	S-09/02/2020-Coewil-26A	3%	4%
	41	Brown Inca	*Coeligena wilsoni*	S-09/02/2020-Coewil-26B	4%	
28	**42**	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-09/02/2020-Helrub-27A	NE	0%

	43	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-09/02/2020-Helrub-27B	NE	
29	**44**	Brown Inca	*Coeligena wilsoni*	S-09/02/2020-Coewil-28A	3%	3%
	45	Brown Inca	*Coeligena wilsoni*	S-09/02/2020-Coewil-28B	IN	
30	**46**	Brown Inca	*Coeligena wilsoni*	S-09/02/2020-Coewil-29A	2%	2%
	47	Brown Inca	*Coeligena wilsoni*	S-09/02/2020-Coewil-29B	IN	
31	**48**	White-booted Racket-tail	*Ocreatus underwoodii*	S-09/02/2020-Ocrund-30A	1%	1%
	49	White-booted Racket-tail	*Ocreatus underwoodii*	S-09/02/2020-Ocrund-30B	NE	
32	**50**	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-09/02/2020-Helrub-31A	NE	0%
	51	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-09/02/2020-Helrub-31B	NE	
33	**52**	Violet-tailed Sylph	*Aglaiocercus coelestis*	S-09/02/2020-Aglcoe-32A	NE	4%
	53	Violet-tailed Sylph	*Aglaiocercus coelestis*	S-09/02/2020-Aglcoe-32B	7%	
	54	White-booted Racket-tail	*Ocreatus underwoodii*	S-09/02/2020-Ocrund-33A	IN	IN
34	**55**	Violet-tailed Sylph	*Aglaiocercus coelestis*	S-09/02/2020-Aglcoe-34A	5%	5%
	56	Violet-tailed Sylph	*Aglaiocercus coelestis*	S-09/02/2020-Aglcoe-34B	5%	
35	**57**	Violet-tailed Sylph	*Aglaiocercus coelestis*	S-09/02/2020-Aglcoe-35A	2%	2%
	58	Violet-tailed Sylph	*Aglaiocercus coelestis*	S-09/02/2020-Aglcoe-35B	1%	
	59	Brown Inca	*Coeligena wilsoni*	S-09/02/2020-Coewil-36A	IN	IN
	60	Brown Inca	*Coeligena wilsoni*	S-09/02/2020-Coewil-36B	IN	
36	**61**	Purple-bibbed Whitetip	*Urosticte benjamini*	S-09/02/2020-Uroben-37A	7%	5%
	62	Purple-bibbed Whitetip	*Urosticte benjamini*	S-09/02/2020-Uroben-37B	3%	
37	**63**	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-09/02/2020-Helrub-38A	1%	1%

	64	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-09/02/2020-Helrub-38B	NE	
38	**65**	Buff-tailed coronet	*Boissonneaua flavescens*	S-10/02/2020-Boifla-39A	1%	2%
	66	Buff-tailed coronet	*Boissonneaua flavescens*	S-10/02/2020-Boifla-39B	2%	
39	**67**	Tyrannine Woodcreeper	*Dendrocincla tyrannina*	S-10/02/2020-Dentyr-40A	2%	2%
	68	Tyrannine Woodcreeper	*Dendrocincla tyrannina*	S-10/02/2020-Dentyr-40B	1%	
40	**69**	Brown Inca	*Coeligena wilsoni*	S-10/02/2020-Coewil-41A	7%	7%
41	**70**	Purple-bibbed Whitetip	*Urosticte benjamini*	S-10/02/2020-Uroben-42A	3%	5%
	71	Purple-bibbed Whitetip	*Urosticte benjamini*	S-10/02/2020-Uroben-42B	7%	
42	**72**	Brown Inca	*Coeligena wilsoni*	S-10/02/2020-Coewil-43A	5%	5%
	73	Brown Inca	*Coeligena wilsoni*	S-10/02/2020-Coewil-43B	IN	
43	**74**	Speckled Hummingbird	*Adelomyia melanogenys*	S-10/02/2020-Ademel-44A	4%	5%
	75	Speckled Hummingbird	*Adelomyia melanogenys*	S-10/02/2020-Ademel-44B	6%	
44	**76**	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-10/02/2020-Helrub-45A	5%	5%
	77	Fawn-breasted Brilliant	*Heliodoxa rubinoides*	S-10/02/2020-Helrub-45B	IN	
45	**78**	Buff-tailed coronet	*Boissonneaua flavescens*	S-10/02/2020-Boifla-46A	6%	9%
	79	Buff-tailed coronet	*Boissonneaua flavescens*	S-10/02/2020-Boifla-46B	11%	
46	**80**	Buff-tailed coronet	*Boissonneaua flavescens*	S-10/02/2020-Boifla-47A	5%	6%
	81	Buff-tailed coronet	*Boissonneaua flavescens*	S-10/02/2020-Boifla-47B	7%	
47	**82**	Buff-tailed coronet	*Boissonneaua flavescens*	S-10/02/2020-Boifla-48A	8%	5%
	83	Buff-tailed coronet	*Boissonneaua flavescens*	S-10/02/2020-Boifla-48B	2%	

Appendix 2.

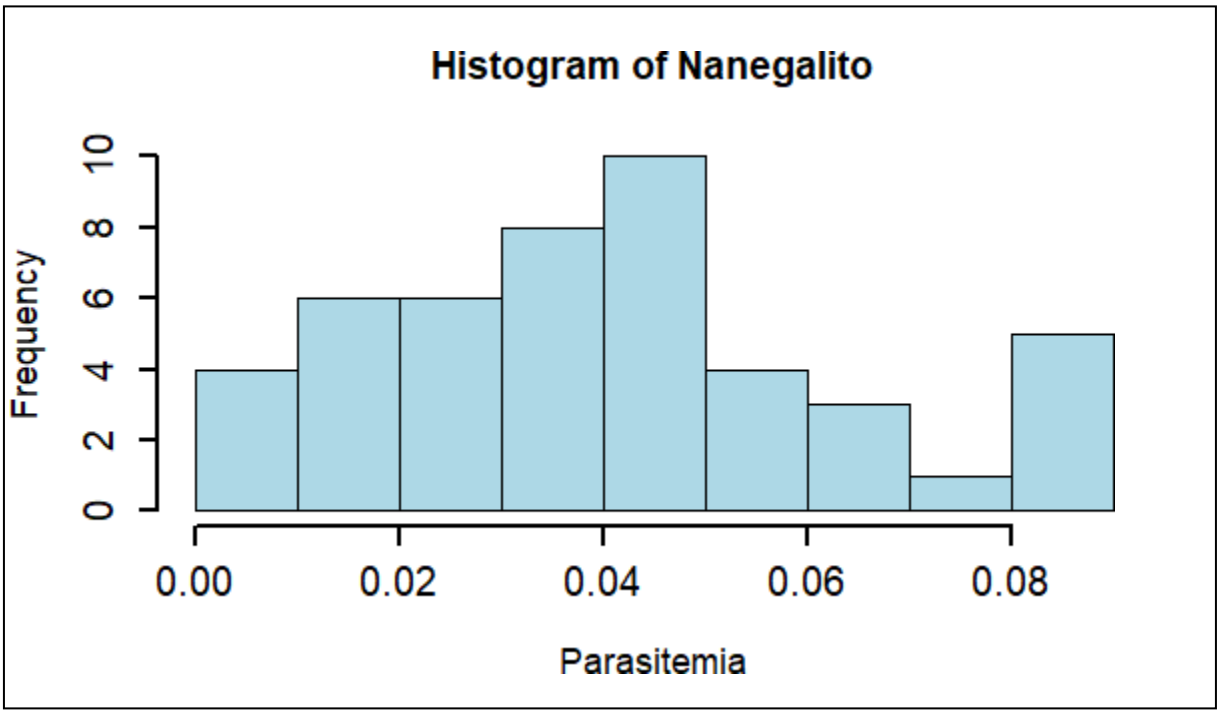
Milpe Bird Sanctuary Sampling Data. A total of 14 birds of 7 species were captured, from that number, one was excluded from the statistical analysis as showed in this table. That sample is marked as NA and colored in red since no blood sample of that individual was obtained.

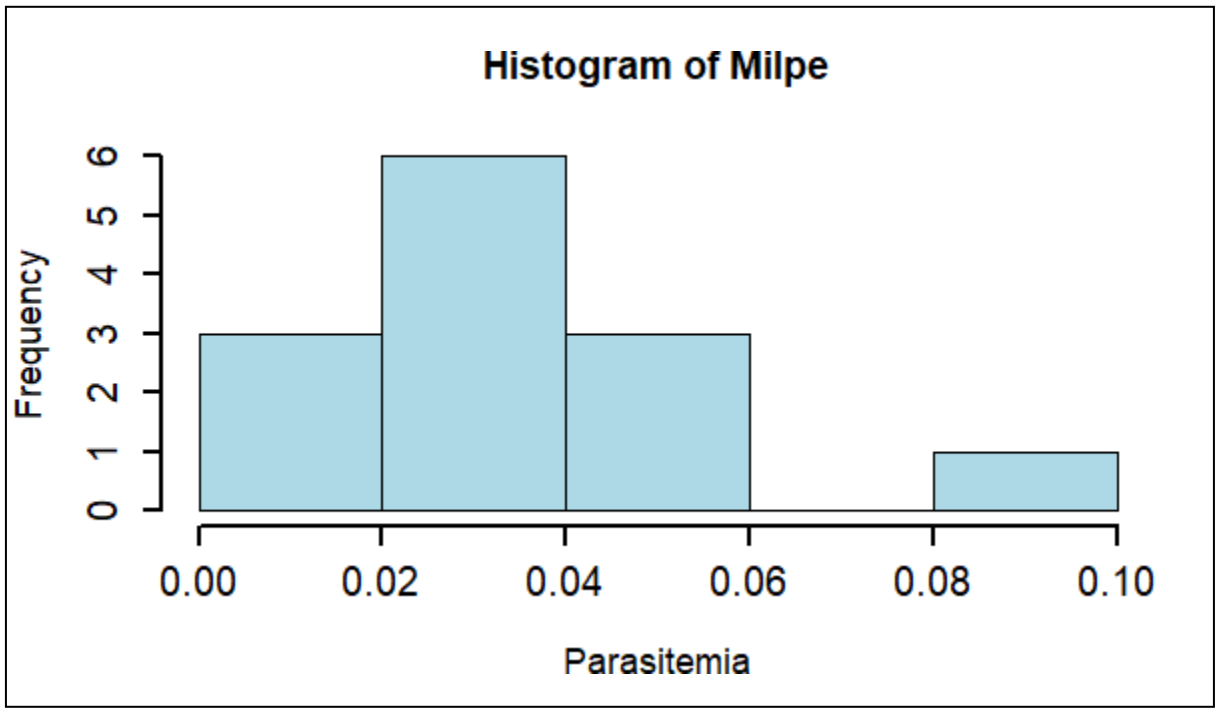
MILPE (FEBRUARY 8th, 2020)						
Individual #	**Sample #**	**Common name**	**Species**	**Code**	**Parasitemia (%)**	**Parasitemia Mean (%)**
48	**1**	Spotted Barbtail	*Premnoplex brunnescens*	S-08/02/2020-Prebru-1A	4%	4%
	2	Spotted Barbtail	*Premnoplex brunnescens*	S-08/02/2020-Prebru-1B	4%	
49	**3**	Club-winged Manakin	*Machaeropterus deliciosus*	S-08/02/2020-Macdel-2A	2%	4%
	4	Club-winged Manakin	*Machaeropterus deliciosus*	S-08/02/2020-Macdel-2B	6%	
50	**5**	Wedge-billed Woodcreeper	*Glyphorynchus spirurus*	S-08/02/2020-Glyspi-3A	4%	3%
	6	Wedge-billed Woodcreeper	*Glyphorynchus spirurus*	S-08/02/2020-Glyspi-3B	1%	
51	**7**	Plain-brown Woodcreeper	*Dendrocincla fuliginosa*	S-08/02/2020-Denful-4A	6%	5%
	8	Plain-brown Woodcreeper	*Dendrocincla fuliginosa*	S-08/02/2020-Denful-4B	4%	
52	**9**	Green-crowned Woodnymph	*Thalurania fannyi*	S-08/02/2020-Thafan-5A	2%	2%
53	**10**	Green-crowned Woodnymph	*Thalurania fannyi*	S-08/02/2020-Thafan-6A	4%	4%
54	**11**	Green-crowned Woodnymph	*Thalurania fannyi*	S-08/02/2020-Thafan-7A	2%	2%
55	**12**	Green-crowned Brilliant	*Heliodoxa jacula*	S-08/02/2020-Heljac-8A	10%	10%
56	**13**	Green-crowned Woodnymph	*Thalurania fannyi*	S-08/02/2020-Thafan-9A	1%	3%
	14	Green-crowned Woodnymph	*Thalurania fannyi*	S-08/02/2020-Thafan-9B	5%	
	15	White-necked Jacobin	*Florisuga mellivora*	S-08/02/2020-Flomel-10A	IN	IN
57	**16**	Green-crowned Brilliant	*Heliodoxa jacula*	S-08/02/2020-Heljac-11A	3%	3%

58	**17**	Green-crowned Brilliant	*Heliodoxa jacula*	S-08/02/2020-Heljac-12A	5%	5%
59	**18**	Green-crowned Brilliant	*Heliodoxa jacula*	S-08/02/2020-Heljac-13A	1%	1%
60	**19**	Green-crowned Woodnymph	*Thalurania fannyi*	S-08/02/2020-Thafan-14A	5%	5%

Appendix 3.

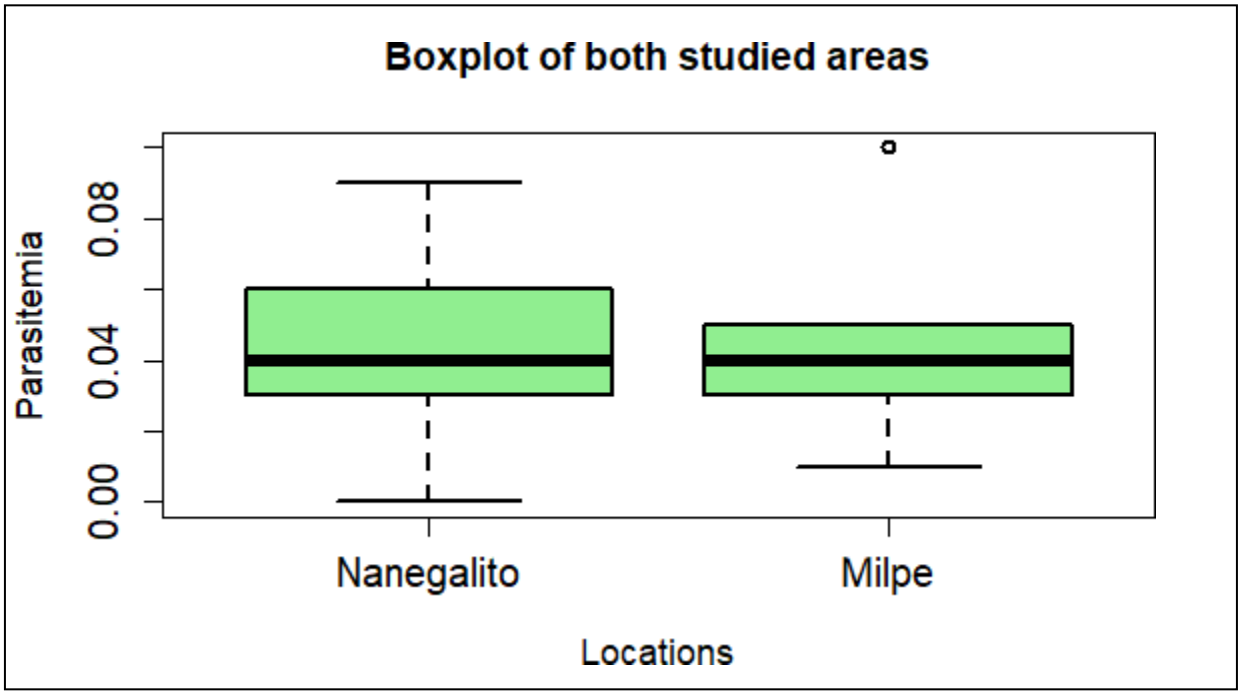
Histograms of the data of the two studied localities, Nanegalito (n1=47) and Milpe (n2=13).

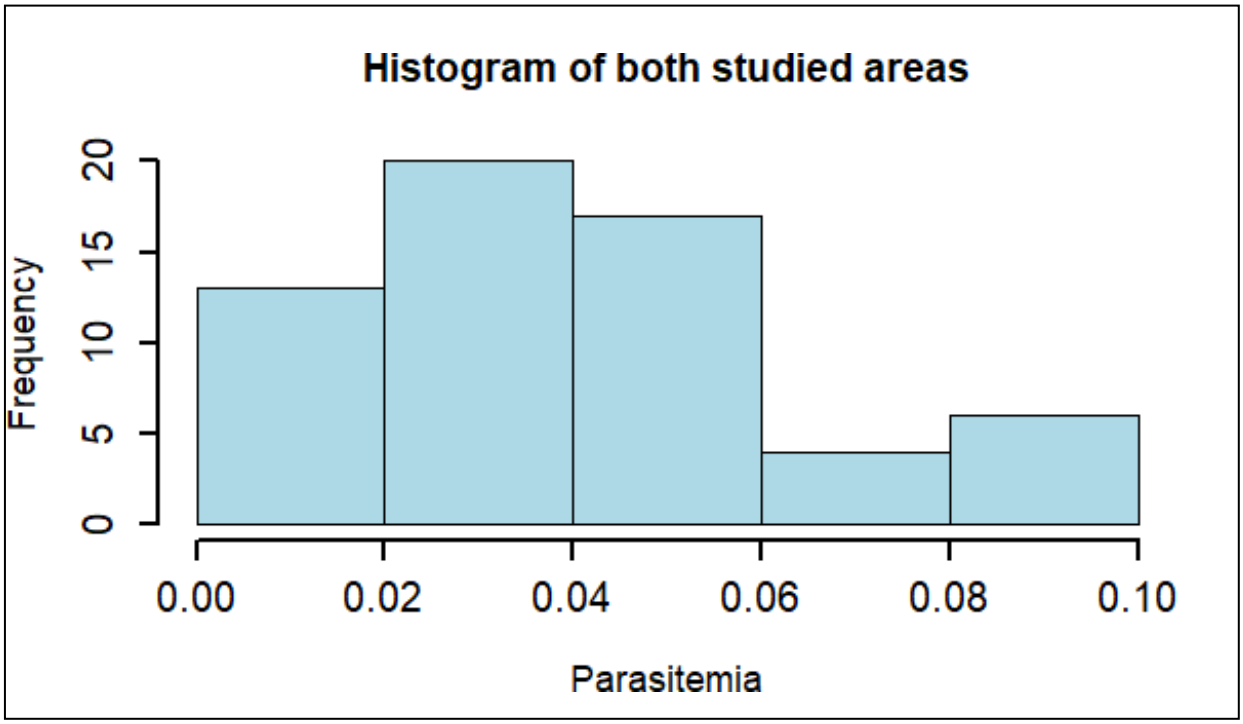




Appendix 4.

Boxplot of the two studied localities, Nanegalito (n1=47) and Milpe (n2=13), and histogram of all the data (N=60).





Appendix 5.

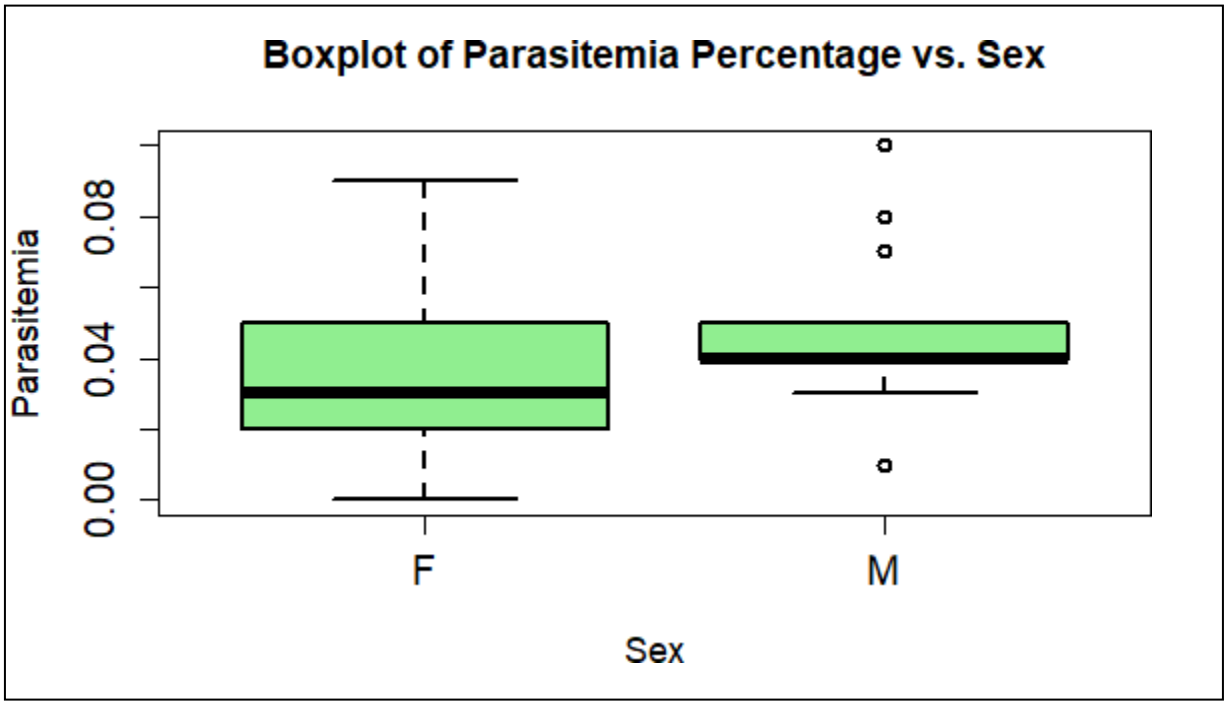
Code used for statistical analysis and p-values obtained from the Shapiro-Wilk and the Mann-Whitney U tests.

```
Data = (c(0.09, 0.06, 0.04, 0.05, 0.04, 0.07, 0.06, 0.08, 0.07, 0.04, 0.09, 0.03, 0.04, 0.05, 0.06,
0.09, 0.04, 0.05, 0.03, 0.03, 0.09, 0.03, 0.03, 0.04, 0.02, 0.02, 0.04, 0.00, 0.03, 0.02, 0.01, 0.00,
0.04, 0.05, 0.02, 0.05, 0.01, 0.02, 0.02, 0.07, 0.05, 0.05, 0.05, 0.05, 0.09, 0.06, 0.05, 0.04, 0.04,
0.03, 0.05, 0.02, 0.04, 0.02, 0.10, 0.03, 0.03, 0.05, 0.01, 0.05))
shapiro.test(Data)
hist(Data)

malaria = read.table(file.choose(), header=T, sep=",")
attach(malaria)
hist(Nanegalito)
hist(Milpe)
boxplot(Nanegalito,Milpe, data = malaria)
help(wilcox.test)
wilcox.test(Nanegalito, Milpe, alt="two.sided", conf.int=T, conf.level=0.95, paired=F, exact=T,
correct=T)
```

```
        Shapiro-Wilk normality test

data:  Data
W = 0.9497, p-value = 0.01504


        Wilcoxon rank sum test with continuity correction

data:  Nanegalito and Milpe
W = 361, p-value = 0.3181
alternative hypothesis: true location shift is not equal to 0
95 percent confidence interval:
 -0.009962243  0.020025771
sample estimates:
difference in location
           0.009999221
```

Appendix 6.

Boxplot of the two studied localities concerning parasitemia vs sex with a total sample size of 37 individuals, 19 females (F), and 18 males (M). Code and Mann-Whitney U test results of the statistical analysis.




```
Parasitemia = read.table(file.choose(), header=T, sep=",")
attach(Parasitemia)
names(Parasitemia)
class(PP)
class(Sex)
help(wilcox.test)
wilcox.test(PP~Sex, alt="two.sided", paired=F, exact=T, correct=T)
```

```
        Wilcoxon rank sum test with continuity correction

data:  PP by Sex
W = 119, p-value = 0.1122
alternative hypothesis: true location shift is not equal to 0
```

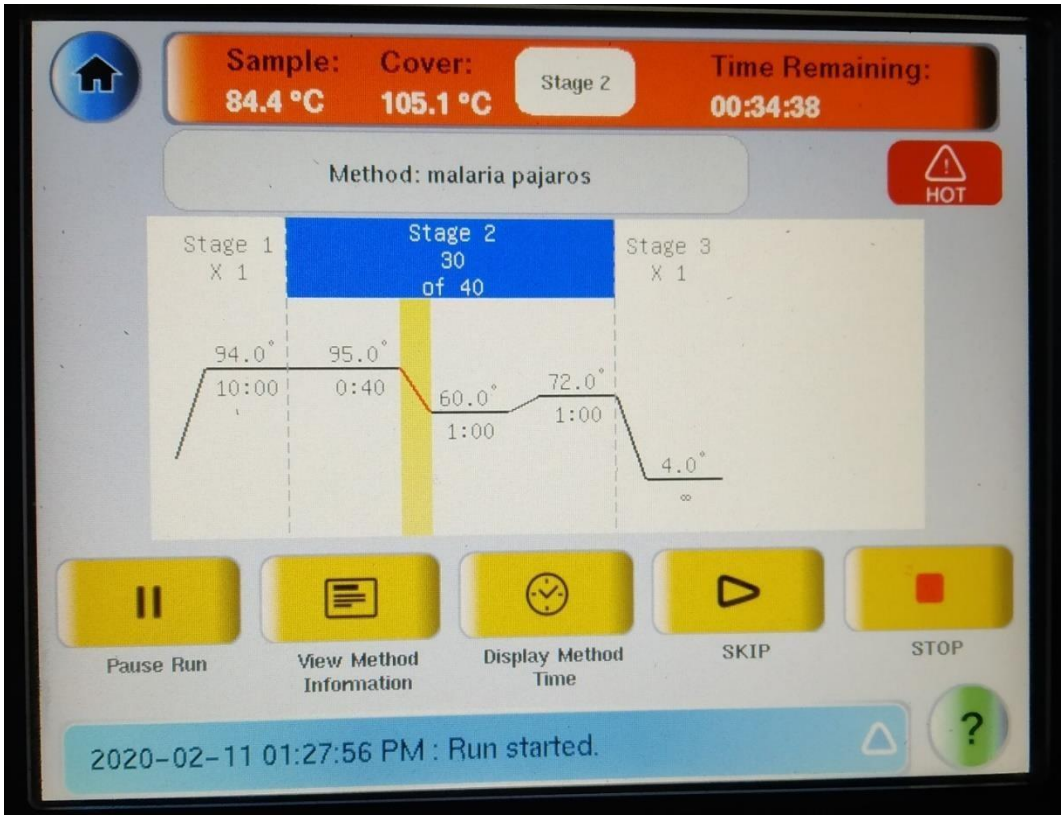
Appendix 7.

Photographs of different bird species captured, sampled, and released during the field trips. (A) *Ocreatus underwoodii* – female, (B) *Heliodoxa jacula* – male, (C) *Aglaiocercus coelestis* – male, (D) *Dendrocincla fuliginosa* – unknown.

Appendix 8.

(A) Polymerase chain reaction standardization parameters used in the thermocycler for DNA amplification. (B) Standardization results of the last PCR run in a 1.5% agarose gel stained with ethidium bromide and visualized under UV light. (1) Molecular ladder. (2)-(5) Hens' DNA. (6) Peafowl DNA. (7) Negative Control – DNA of a noninfected human patient.

(A)



(B)