

# Evaluation of Avian Malaria in An Inter-Andean Dry Forest of Imbabura Province in Ecuador

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## Research

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## RESEARCH

# Evaluation of avian malaria in an inter-Andean dry forest of Imbabura Province in Ecuador

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## Abstract

**Background:** Hematozoa of the genus *Plasmodium* cause malaria in birds, and are frequently found in passerines. They are characterized by particularly small meronts and elongated gametocytes in peripheral blood. Although the disease is not fatal, it affects reproductive success and, therefore, the biodiversity of the birds, so it is a necessity to know the species of birds that are infected.

**Methods:** The presence of *Plasmodium* in passerine birds were investigated in three locations in the inter-Andean dry forest of the province of Imbabura in Ecuador. Prevalence and parasitaemia were determined by light microscopy of blood smears and was confirmed by the indirect immunofluorescence assay (IFA) with a monoclonal antibody that recognizes protein 1 of merozoite surface (MSP-1).

**Results:** The percentage of prevalence and parasitaemia in the bird species captured in the three locations was determined and was related to the elevation and presence of bodies of water in the studied areas. A 100% prevalence was found at the lowest site but decreased with elevation and the absence of nearby water bodies. The IFA test supported the identity of the *Plasmodium* parasite.

**Discussion:** This study shows that bird species are highly parasitized in the dry forest of Imbabura and underscores the influence elevation and the presence of water as a possible source proliferation of the vector insects. As elevation increases, prevalence and parasitaemia decrease. Besides, we present a useful new diagnostic tool, a monoclonal antibody that recognizes *Plasmodium* protein MSP-1, which is highly conserved among the species of this parasite.

**Keywords:** Avian malaria; Imbabura; dry forest; *Plasmodium*; monoclonal antibody; MSP-1

## Background

Hematozoa of the genus *Plasmodium* cause malaria in birds. They are frequently found in passerine birds along with other avian hemosporidians in the genus *Leucocytozoon*. Ten species of the parasite have been described, characterized by small meronts and elongated gametocytes found in the peripheral blood [1, 2]. Along with other haemosporidians, such as *Haemoproteus* and *Leucocytozoon*, *Plasmodium* is known to affect entire bird communities [3]. For instance, *P. relictum* has caused severe consequences in some endemic bird species of Hawaii [3].

The picture of avian *Plasmodium* biogeography and ecology in the Neotropics is as of yet still incomplete. Most published studies take either one of two approaches: 1) detection of haemoprotean parasites in bird communities in different areas or 2)

geographic patterns of avian malaria in focal avian host species. Both approaches differ in scope, but some of the conclusions coincide. At the regional scale, most studies focus on prevalence (proportion of infected individuals) of avian malaria rather than levels of parasitaemia.

The prevalence of malaria in bird communities varies widely. Several environmental and bird species-specific traits appear to explain prevalence partially. Environmental variables associated with prevalence in some studies are temperature, rainfall, and elevation [4] as well as vegetation structure [5, 6, 7]. Among avian species-ecological traits are migratory status, nest type, social behavior, and size [5, 8], although the supporting evidence is often lacking [9]. Other factors that likely affects the prevalence of malaria in birds are seasonality and spatial distribution of culicid mosquito vectors in the landscape [10, 11, 12].

Previous studies in the Andes of Ecuador, where the biodiversity of flora and fauna is exceptionally high, suggest that avian malarial parasites are well diversified and more common at higher elevations than at lower ones [6]. At first, this outcome appears counterintuitive, but given that the *Plasmodium* is represented by many lineages that are more or less specific to their host [1, 13, 14], each lineage is faced with a lower probability of encountering a potential host, thus diminishing infection rates [6].

Two species that have been studied in detail are *Zonotrichia capensis* [15, 16] and *Troglodytes aedon* [13]. *Zonotrichia capensis* hosts a great diversity of *Plasmodium* throughout its entire range. Diversity is highest at low elevations. Prevalence averages 25% with a range of 0-100%. The temperature may be the most limiting factor for *Plasmodium* that infect *Z. capensis* [16]. In western Peru, *Plasmodium* prevalence peaked at mid-elevations, although the prevalence was generally low [15].

Galen and Witt (2014) found a great diversity of haemoprotean parasites in the *T. aedon* that does not reflect the deep population structure of the avian host. In some, when populations the parasites nonetheless show a distinct pattern of species replacement along an elevational gradient that might be related to temperature and precipitation.

As we are still trying to understand patterns in prevalence and parasitaemia of *Plasmodium*, climate change is now allowing the establishment of insect vectors in previously unoccupied areas, facilitating the transmission of the parasite to immunologically naïve animals [2, 15]. Altitude and latitude create large-scale patterns in vector distributions. Vectors tend to be geographically restricted by temperature and rainfall, and both of these environmental variables are subject to change with global warming [15, 17]; therefore, many bird species are at risk due to the spread of the malaria vector especially into higher elevation areas [18].

This study aimed to determine the prevalence and parasitaemia of *Plasmodium* in the birds of a tropical dry, inter-Andean forest in the province of Imbabura. To account for differences associated to elevation, we sampled birds at 1580 meters above sea level (m.a.s.l) and near 2050 m.a.s.l. Furthermore, we wanted to confirm the presence of *Plasmodium* using immunofluorescent monoclonal antibodies to Merozoite Surface Protein 1 (MSP-1) [19].

## Material and Methods

### Study area

The area of study was the upper, southern section of the Chota Valley in the Canton of San Miguel de Urcuquí, Imbabura province, Ecuador. The native ecosystem is a dry inter-Andean forest, but presently the landscape is dominated by a matrix of agricultural lands mixed with native scrub. The average annual temperature is 20 °C, and annual rainfall is 623 mm. Birds were caught in three localities to collect blood for blood smears: Salinas at 1580 m.a.s.l., Las Yunguillas at 2050 m.a.s.l., and the proximities to the Yachay Tech University at 2065 m.a.s.l (henceforth referred to as Yachay). The locations of Salinas and Las Yunguillas are in the vicinity of a small river and irrigation canal, respectively. There was no water source near the Yachay location. Also, we sampled birds at 2460 m.a.s.l. in an area called El Hospital. Here we captured only 12 birds, but due to the low sample size, this group was not included in the statistical analyses.

### Bird capture and blood sampling

We captured wild hummingbirds and passerines through mist nets. A blood sample was taken by puncturing the brachial vein. One to three drops of blood were used for the smears, which were taken in duplicates if enough blood was available. After taking the blood sample and identifying the species of birds, they were released back into their environment. The blood smears were air-dried, fixed with absolute methanol in the field, and stained with Giemsa's reagent in the laboratory.

### Determination of parasitaemia

The staining of smears was achieved with the Giemsa's reagent in PBS solution at 7.0 pH for 5 minutes. An optical microscope (Leica DM 300 model) set at 1000x with immersion oil was used to determine parasitaemia and parasite, merozoite/ring, and schizont stages [1]. Valkiūnas, Iezhova, and collaborators have presented recommendations for the identification of avian malaria on smears under the light microscope [1, 20].

The fields of view in the microscope contained around 200 RBC/field in each sample. We calculated the percentage of parasitaemia as the number of infected RBC per 100 cells [20, 21].

### Indirect immunofluorescence Assay (IFA)

Although we only determine the infection by morphological identification with optical microscopy, we confirmed the identity of *Plasmodium* by indirect immunofluorescence (IFA) using a monoclonal antibody that recognizes the 19 KDa fraction of the Merozoite Surface Protein (MSP-1) of *Plasmodium yoelii*. Since this protein is conserved in all *Plasmodium* species, fluorescence confirms the presence of this parasite [19].

The IFA was performed at room temperature in a wet chamber, and one smear of the three prepared in the field was acetone-fixed. After the mAb F5 was placed on the blood smear as the primary antibody (diluted in PBS 1:100), it was incubated for 1 hr at 37°C. After incubation, the slides were washed three times in PBS. The negative control was applying only PBS solution to the slide. Later the slides

were incubated for 1 hr in fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (IgG; Sigma) diluted in PBS 1:1000. Preparations were examined with a fluorescence microscope (600X), photographed, and analyzed with Optika-View7 software [22].

Finally, we studied the use of a mAb that recognizes the MSP-1 of *Plasmodium yoelii*, a species of mouse parasite. We analyzed the sequences reported in the literature to explain the use of this antibody by recognizing highly conserved sequences between different *Plasmodium* species and allowing us to confirm the diagnosis in blood samples of the birds.

### Statistical analysis

A Will-Shapiro test was applied to verify the normality of the parasitaemia data. If normality was supported, one-way Analysis of Variance (ANOVA) was used to test for differences among sites. In contrast, when the data were not normally distributed, a Kruskal and Wallis test was used [23, 24, 25, 26]. Finally, an Exact Wilcoxon Mann-Whitney Rank Sum Test was implemented to test for pairwise differences between sites. R-Studio (2015) Version 1.1.456 was used for all of the statistical procedures [27].

## Results

A total of 86 birds were captured in the three areas. Among the areas studied, the one with the enormous diversity was Yunguillas, with 13 different species of birds, followed by Salinas with seven species of birds and Yachay with four species.

At Salinas, all 30 birds were infected. The highest level of parasitaemia of 8% was found in a *Myiophobus fasciatus* and a *Saltator striatipectus*. At Las Yunguillas, 25 birds were captured, of which 92% were infected. The species with the highest percentage of parasitaemia was *Turdus maculirostris* at 4.2% and *Z. capensis* at 3.8%, followed by *Conirostrum cinereum* with 3.2% parasitaemia. At Yachay, 19 birds were captured, with a prevalence of 37%. The species with the highest parasitaemia were *T. aedon* at 3.2%, *Tiaris olivacea* and *Z. capensis* with 2.5% and 2.2%, respectively (Figure 1). Average parasitaemia levels were highest at Salinas, and lowest at Yachay (Table 1).

At the El Hospital site, 12 birds were captured, of which nine were infected, representing a prevalence of 75%. Parasitemia was less than 1% in all birds. The species with the highest parasitaemia at 0.05% was *Z. capensis*.

Table 1 and the Figure 2 show the differences among the three locations with respect to parasitaemia percentage.

As the data of parasitaemia levels were not normally distributed (Wilk-Shapiro Test;  $p_i < 0.0001$ ) a Kruskal and Wallis test was used to compare the average parasitaemia at the three sites. Sites were significantly different ( $p = 0.003418$ ), due to significantly lower levels of parasitaemia at Yachay (Table 1). Yachay was the only site without a nearby source of water.

We confirmed that *Plasmodium* caused the infection observed in the birds' blood with an Indirect Immunofluorescence Assay (IFA), using as a primary antibody a F5 mAb that recognizes the 19 kDa fragment of MSP-1 [19]. Ring stage (trophozoite) within the bird's erythrocyte and macrogametocyte stage are visible [28]. Fluorescence of mAb F5 recognizing the surface of a merozoite confirms that the parasite in question is *Plasmodium* (Figure 3D).

## Discussion

### Prevalence and parasitaemia

Prevalences of *Plasmodium* at two of the study sites (Salinas and Las Yunguillas) were unexpectedly high for a dry environment with 92% and 100%, respectively. High prevalence can be explained by the nearby presence of water in the form of a river and an irrigation channel, respectively. A much lower prevalence at the third site (37%) is reflective of the fact that nobody of water is in the vicinity. The site with the highest prevalence was the lowest and presumably warmest site. In parallel to the observed pattern in prevalence, levels of parasitaemia were highest at Salinas and lowest at Yachay. Interestingly, *T. aedon* and *Z. capensis*, two heavily studied species [13, 15, 16], do not show a clear trend with elevation (Figure 1).

The elevations of the sites of Las Yunguillas and Yachay are very similar. Here the difference was the presence of an irrigation canal only found at Las Yunguillas. As a result, at Las Yunguillas, more than twice as many infected individuals of birds were caught than at Yachay. However, the maximum percentages of parasitaemia were similar at 4.2% and 3.2%, respectively. The presence of water undoubtedly favors the reproduction of the vector mosquito and increases the transmission of the disease [29, 30].

Also, between the locations of Las Yunguillas/Yachay (2065 m.a.s.l.) and Salinas (1580 m.a.s.l.), there is a 485-meter elevation difference that, considering a temperature lapse rate of  $6.88^{\circ}\text{C km}^{-1}$  [31], would imply a temperature difference of about  $3.36^{\circ}\text{C}$ . Since the zone of Salinas has a slightly warmer climate and has bodies of water in the area, conditions could be favorable for multiple reproductive cycles of the definitive dipteran hosts [32].

Comparing the locations of Las Yunguillas and Salinas with the site of El Hospital, which is located at 2460 m.a.s.l. and next to an irrigation canal (representing a 900 m elevational difference with Salinas and 400 m with Las Yunguillas), there appears to be a further decrease in prevalence and parasitaemia at the higher location. At El Hospital, only 75% of the birds captured were infected, with very low percentages of parasitaemias of around 0.05%. These suggest that although there is a body of water, altitude can influence the transmission of the disease as reported by several researchers in Hawaii and South Africa [29, 30].

It is important to note that mild cases of malaria, as inferred by low levels of parasitaemia, do not necessarily imply lower levels of virulence. Birds with low parasite loads could still experience mortality due to pharenozoites in the brain, as reported by a histological study on birds that were experimentally infected with *P. homocircumflexum* [33].

### Indirect immunofluorescence Assay (IFA)

The MSP-1 protein is anchored to the parasite's membrane, during the formation of the merozoite, through the carboxyl terminus of the glycosyl phosphatidyl inositol GPI protein. The carboxyl end of the GPI protein could be identified in *P. relictum* (lineage P-SGS1)[34], and this region is conserved between *P. falciparum*, *P. gallinaceum* and *P. relictum* (lineage P-SGS1) with the characteristic motif "FCSSS" of amino acids [35, 36]. This "FCSSS" motif is highly conserved among all species of haematozoa, including avian malaria species. In the avian malaria p19 peptide,

it was also possible to find the two epidermal growth factor (EGF) domains, based on the six conserved cysteine residues [37]. In *P. falciparum*, cysteine residues form disulfide bridges that are recognized by the immune system and produce a protective host antibody response against the parasite. In some parasites, the antibody response against the p19 epitopes can cross-react in different malaria species and lineages [38, 39].

In this system, the MSP-1 peptide p19 could be an excellent target for investigating cross-immunity between different species, and the change in this cross-reactivity will depend on the phylogenetic distance between the parasites. Besides, *P. relictum* has caused massive population declines and mortality in endemic communities of birds where it has been introduced, especially in previously malaria-free populations (for example, Hawaii) [40]. Here, MSP1-p19 could be used as a candidate gene to investigate whether individual birds surviving malaria infection produce antibodies against this peptide, to examine whether the various host species carrying the parasite follow the same mechanisms and metabolic pathways in the fight against the disease [40].

## Conclusions

In this study, we determined the prevalence and parasitaemia in bird species infected with avian malaria in the dry forest ecosystem. Both of these measures decrease with elevation. Also, the presence of irrigation channels or rivers in the locality increases the transmission of the disease in birds, since the vector requires water for reproduction. Climate change likely will increase prevalence and parasitaemia in the future at higher elevations. We recommend that parasitaemia be considered in studies on the distribution of avian malaria as prevalence does not give us the whole picture.

In addition, this is the first work where a monoclonal antibody that recognizes the MSP-1 molecule from mammalian *Plasmodium* has been used as a diagnostic tool in birds since this monoclonal antibody recognizes the conserved regions of merozoite surface protein 1 in all higher vertebrates, suggesting that it is an excellent tool for diagnosis of malaria in a great range of vertebrates.

**Ethics approval and consent to participate.** Not applicable

**Consent for publication.** Not applicable

**Availability of data and material.** Not applicable

### Competing interests

The authors declare that they have no competing interests.

**Funding.** Not applicable

### Author's contributions

LS contributed to study design, bird capture, blood sampling, determination of parasitaemia, data analysis, the performance of the Immunofluorescence assays, and manuscript writing. JC contributed to bird capture, sampling, and statistical analysis. MT contributed to study design, bird capture and identification, sampling, and final preparation of the manuscript. NSV contributed to the DNA sequence analysis of MSP-1 in other *Plasmodium* species, and IRA contributed to the analysis of the data and drafting the manuscript. SA contributed to bird capture, sampling, slides Giemsa preparation, and edited the manuscript. DC, MA, and MJA contributed to bird capture, sampling, and slides Giemsa preparation. All authors read and approved the manuscript.

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### Author information

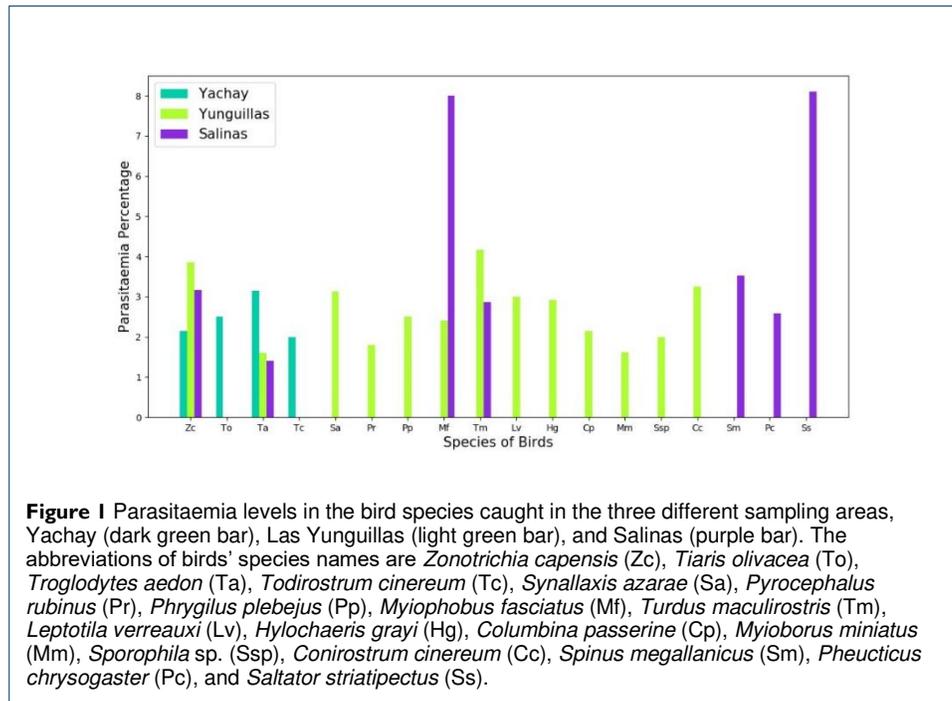
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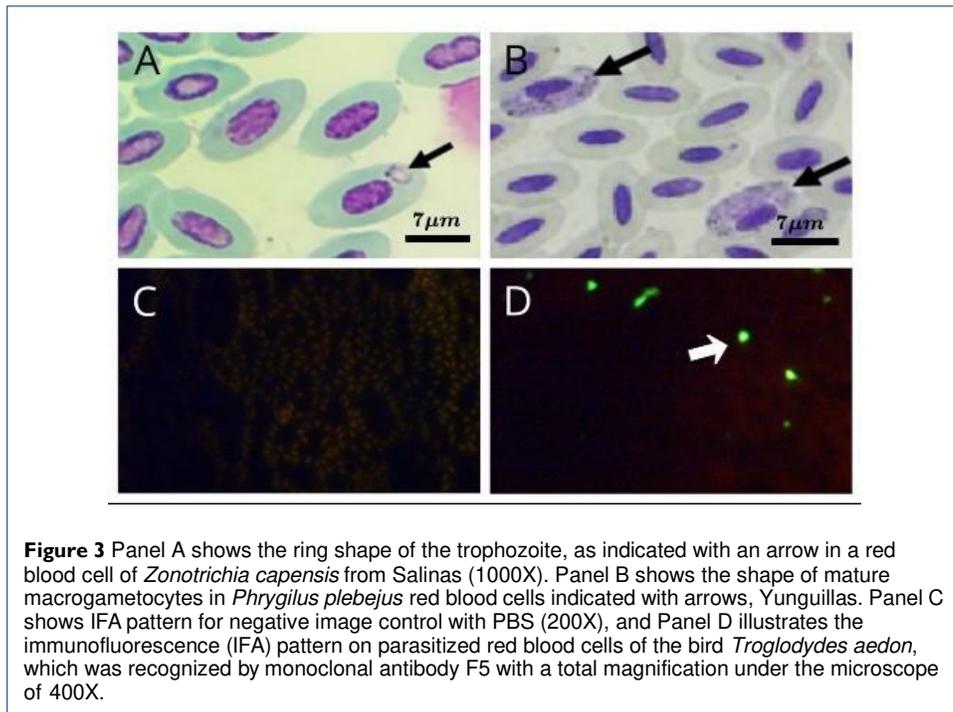
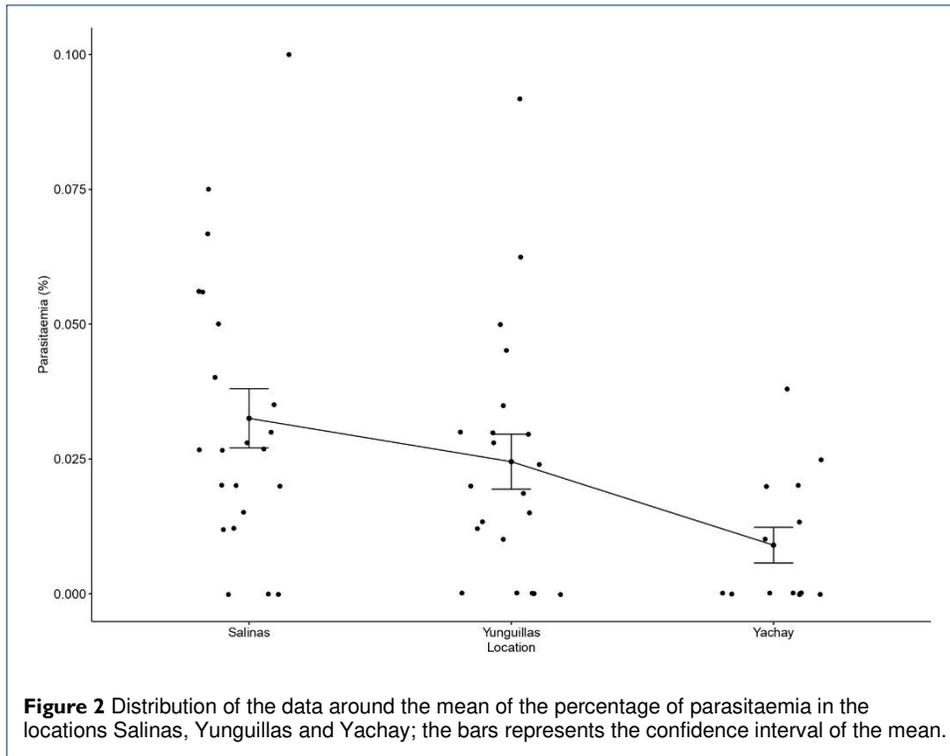
**Figures**



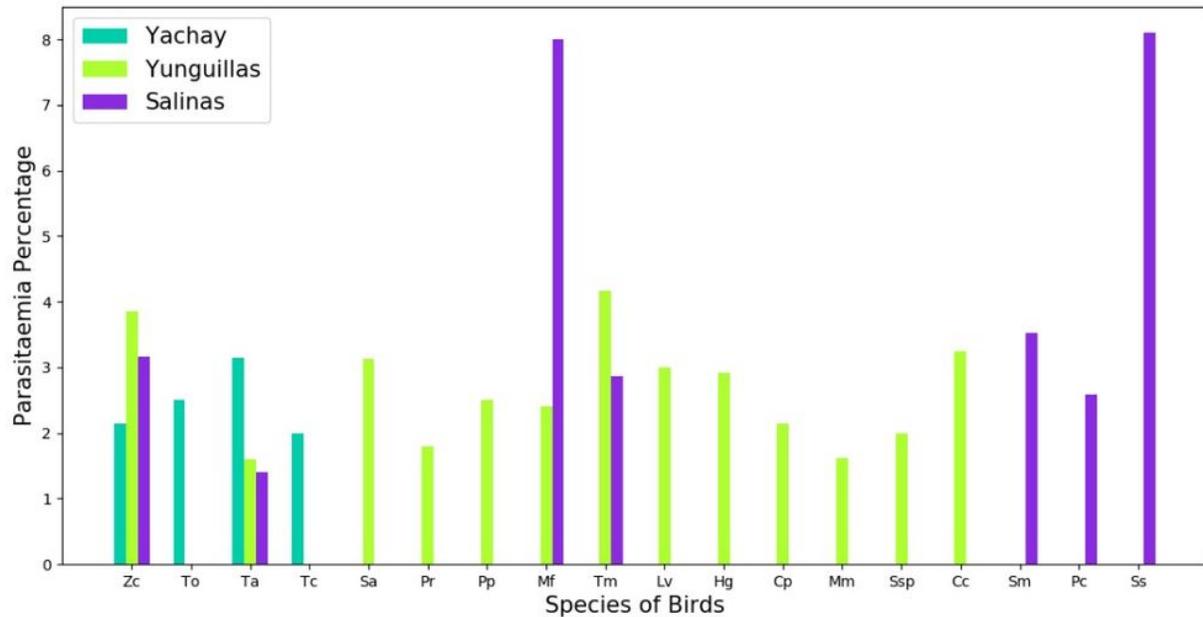
**Tables**

**Table I** P-values for pairwise comparison of parasitaemia at the three study sites.

	Salinas	Yunguillas
Yunguillas	0.2885	1.0
Yachay	0.0055	0.0421

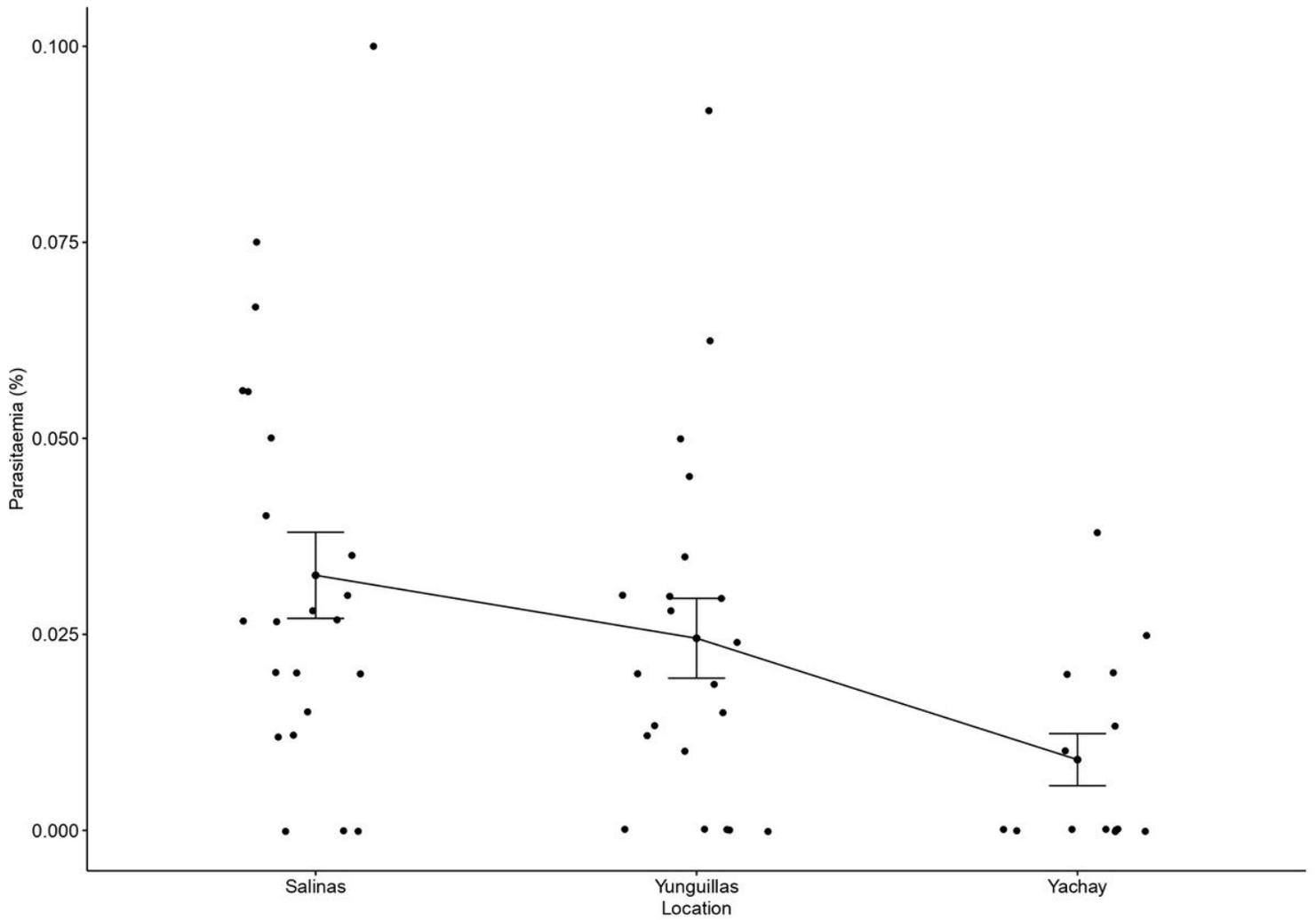


# Figures



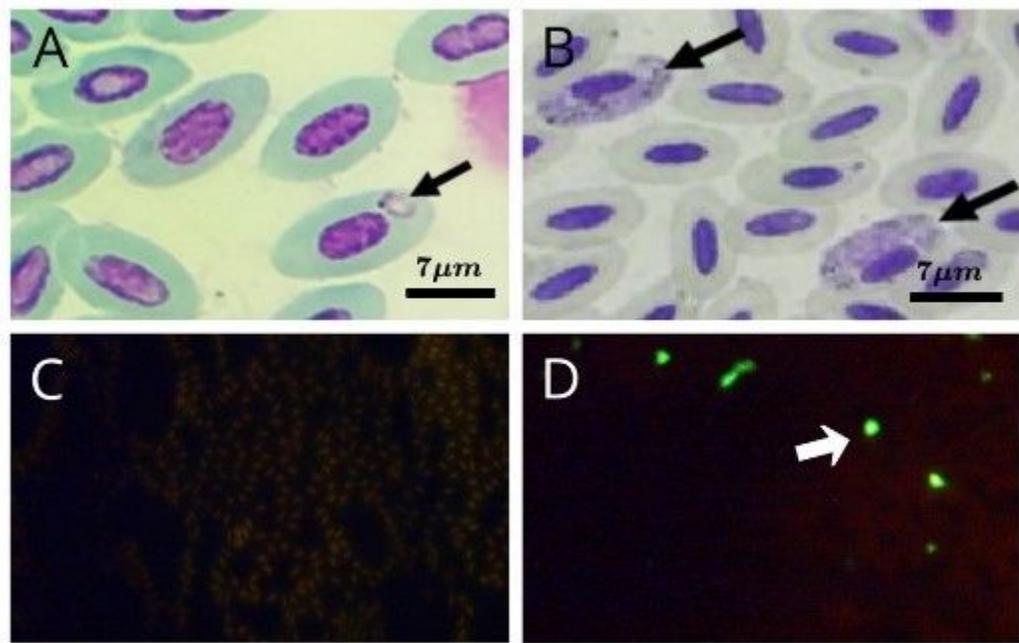
**Figure 1**

arasitaemia levels in the bird species caught in the three different sampling areas, Yachay (dark green bar), Las Yunguillas (light green bar), and Salinas (purple bar). The abbreviations of birds' species names are *Zonotrichia capensis* (Zc), *Tiaris olivacea* (To), *Troglodytes aedon* (Ta), *Todirostrum cinereum* (Tc), *Synallaxis azarae* (Sa), *Pyrocephalus rubinus* (Pr), *Phrygilus plebejus* (Pp), *Myiophobus fasciatus* (Mf), *Turdus maculirostris* (Tm), *Leptotila verreauxi* (Lv), *Hylochaeris grayi* (Hg), *Columbina passerine* (Cp), *Myioborus miniatus* (Mm), *Sporophila sp.* (Ssp), *Conirostrum cinereum* (Cc), *Spinus megallanicus* (Sm), *Pheucticus chrysogaster* (Pc), and *Saltator striatipectus* (Ss).



**Figure 2**

Distribution of the data around the mean of the percentage of parasitaemia in the locations Salinas, Yunguillas and Yachay; the bars represents the confidence interval of the mean.



**Figure 3**

Panel A shows the ring shape of the trophozoite, as indicated with an arrow in a red blood cell of *Zonotrichia capensis* from Salinas (1000X). Panel B shows the shape of mature macrogametocytes in *Phrygilus plebejus* red blood cells indicated with arrows, Yunguillas. Panel C shows IFA pattern for negative image control with PBS (200X), and Panel D illustrates the immunofluorescence (IFA) pattern on parasitized red blood cells of the bird *Troglodytes aedon*, which was recognized by monoclonal antibody F5 with a total magnification under the microscope of 400X.