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# EFFECTS OF CLIMATE AND LAND USE ON DIVERSITY, PREVALENCE, AND SEASONAL TRANSMISSION OF AVIAN HEMATOZOA IN AMERICAN SAMOA

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## ABSTRACT

The indigenous forest birds of American Samoa are increasingly threatened by changing patterns of rainfall and temperature that are associated with climate change as well as environmental stressors associated with agricultural and urban development, invasive species, and new introductions of avian diseases and disease vectors. Long term changes in their distribution, diversity, and population sizes could have significant impacts on the ecological integrity of the islands because of their critical role as pollinators and seed dispersers. We documented diversity of vector borne parasites on Tutuila and Ta'u Islands over a 10-year period to expand earlier observations of *Plasmodium, Trypanosoma,* and filarial parasites, to provide better parasite identifications, and to create a better baseline for detecting new parasite introductions. We also identified potential mosquito vectors of avian *Plasmodium* and *Trypanosoma*, determined whether land clearing and habitat alterations associated with subsistence farming within the National Park of American Samoa can influence parasite prevalence, and determined whether parasite prevalence is correlated with seasonal changes in rainfall, temperature and wind speed.

Three taxonomically distinct lineages of *Plasmodium* were identified from mosquito vectors and forest birds based on partial sequence data from parasite mitochondrial genes. All three have been described from passerine and galliform birds in Australasia. Two lineages, SCEDEN01 and ORW1, had elongate gametocytes and large schizonts that were consistent with species of *Plasmodium* in the subgenus Giavannolaia, but were taxonomically distinct from known morphological species of *Plasmodium* based on a Bayesian phylogenetic analysis of a 478 bp region of the parasite cytochrome b gene. Both are candidates for description as new species. The third lineage (GALLUS02) was detected only in mosquito vectors on Tutuila and was similar in cytochrome b sequence to *P. juxtanucleare*, a pathogenic species of *Plasmodium* from chickens and other galliform birds from Australasia, Africa, and South America. *Plasmodium relictum*, the malarial parasite that has had such a devastating impact on Hawaiian forest birds, was not detected. We observed large, striated trypanosomes in avian hosts from both Tutuila and Ta'u Islands that fell within the same taxonomic clade as *T. corvi* and *T. culicavium* based on 18S ribosomal DNA sequence. We also observed sheathed microfilariae with pointed tails that had some morphological similarities to microfilaria from species of *Pelecitus*, *Struthiofilaria* and *Eulimdana*, but identification will require recovery and examination of adult filarial worms from the connective tissue or body cavities of infected birds. We also observed one or more species of haemococcidians (*Isospora*, synonym = *Atoxoplasma*) within circulating lymphocytes from multiple avian host species.

Overall prevalence of *Plasmodium* was higher on Ta'u (22%, 75/341) than Tutuila (9.2%, 27/294), with most infections occurring in Polynesian starlings, Samoan starlings, Wattled honeyeaters, and Cardinal honeyeaters. Prevalence was relatively constant from year to year and between seasons at individual study sites, but varied among study sites, with highest rates of infection in areas with agricultural activity at Faleasao (37.4%, 73/195, Ta'u Island) and Amalau Valley (9.7%, 21/216, Tutuila Island). Prevalence in more remote areas of the National Park of American Samoa was lower, ranging from 1.4% (2/146) at Laufuti and Luatele on Ta'u to 7.7% (6/78) at Olo Ridge on Tutuila. Similar trends were evident for infections with *Trypanosoma* and filarial worms. Overall prevalence was not influenced significantly by warmer, wet (summer) or cooler, dry (winter) season.

We detected *Plasmodium* infections in *Culex sitiens* and *C. quinquefasciatus* through either salivary gland and midgut dissections or PCR amplification of parasite cytochrome b genes in pooled or individual samples of mosquitoes that were collected on Tutuila. Pooled or individual *Aedes oceanicus*, *A. polynesiensis*, *A. tutuilae*, *A. upolensis*, *A. nocturnus*, *Aedes* (*Finlaya*) (mixed pools of *A. samoanus*, *A. oceanicus*, *A. tutuilae*), *Aedes* (*Stegomyia*) (mixed pools of *A. aegypti*, *A. upolensis*, *A. polynesiensis*), and *C. annulirostris* were negative for *Plasmodium*, but we detected infections with *Trypanosoma* through midgut and salivary gland dissections in a single *C. sitiens* from Amalau Valley, Tutuila and three *A. oceanicus* from Faleasao, Ta'u. Two of the *A. oceanicus* from Faleasao amplified successfully with *Trypanosoma* primers, but sequences were distinctly different from those obtained from avian hosts.

We found a strong association between land use and prevalence of mosquito-transmitted parasites on Ta'u Island with odds of being infected more than 20 times greater in agricultural plots than more remote native forest. This relationship was evident on Tutuila Island but not statistically significant because of the close proximity of study sites and observed movement of birds between native forest and agricultural land. Our data support previous studies that have suggested that *Plasmodium* and other vector-borne parasites are part of the indigenous parasite fauna in American Samoa. Transmission dynamics appear to be affected by environmental changes associated with land use practices.

## INTRODUCTION

Located in the central South Pacific, the Samoan archipelago is composed of the larger western islands of independent Samoa and the five eastern volcanic islands and two coral atolls that comprise the unincorporated territory of American Samoa. While politically divided, these island communities share a common cultural identity, biological heritage, and an indigenous avifauna that is vulnerable to the effects of an expanding human population, habitat degradation, invasive species, novel disease introductions, catastrophic natural disasters, and limited natural habitat (Utzurrum et al. 2006).

The diversity of extant land birds in American Samoa reflects common families and genera that have been reported on other SW Pacific Island archipelagoes (Steadman 2006) and includes two species of honeyeaters (Meliphagidae), two species of starlings (Sturnidae), four species of doves (Columbidae), three species of rails (Rallidae), a kingfisher (Alcedinidae), a heron (Ardeidae), a duck (Anatidae), a monarch flycatcher (Monarchidae), a parrot (Psittacidae) and a swiftlet (Apodidae) (Table 1). Some of these are potential endemic species or subspecies based on recent morphological and molecular analyses (e.g., *Clytorhynchus vitiensis powelli*, *Foulehaio carunculata*, and *Aplonis tabuensis manuae*) and include region-wide uncommon to rare species (e.g., *Gallicolumba stairi* and *Porzana tabuensis*) (Utzurrum et al. 2006, Watling 2001, Andersen et al. 2014). Many of the species contribute to long-term forest ecosystem stability as flower pollinators and seed dispersers (Trail 1994, Webb et al. 1999). Although populations of most species are presently stable (Seamon et al. 2010), the number of extant species in American Samoa is a little more than half of what is currently present in independent Samoa. This may be a reflection of declines and range contractions associated with colonization of the archipelago by early Polynesians (Steadman 2006). In comparison to Hawai`i, however, the historical avifauna of American Samoa has remained relatively intact, with the possible loss of only one modern forest bird species (Mao, *Gymnomyza samoensis*, still extant in independent Samoa).

Previous studies have documented the presence of hemococcidians (*Isospora,* synonym = *Atoxoplasma*) (Adamczyk et al. 2004) and at least three mosquito-borne hematozoan parasites in forest bird communities in American Samoa. These include filarial worms, at least one species of trypanosome (*Trypanosoma* sp.) similar to *T. avium*, and at least one species of *Plasmodium* with morphological similarities to *P. circumflexum* (Jarvi et al. 2003, Atkinson et al. 2006). Relatively high prevalence of infection and absence of overt pathogenicity suggests that these parasites may be indigenous to the archipelago, but region wide surveys are lacking and relatively little is known about their identity and distribution throughout the SW Pacific. Given the long term threats that endemic and indigenous forest birds in American Samoa face from both environmental and anthropogenic stressors, it is possible that transmission dynamics of the existing parasite community may be altered through host shifts, introductions of new parasites or vectors, land use changes that affect vector populations, and changing temperature and rainfall patterns, with subsequent detrimental effects on forest birds. We sampled forest bird populations periodically between 2001 and 2011 to collect detailed information about prevalence, host and habitat associations, vectors and genetic and morphological diversity of *Plasmodium*, *Trypanosoma*, and filarial parasites on Tutuila and Ta`u islands. Our goals were to 1) document existing parasite diversity by molecular and morphological methods, provide better identifications of species of *Plasmodium* and *Trypanosoma*, and establish a firm baseline for detecting new parasite introductions, 2) identify potential mosquito vectors of avian *Plasmodium* and *Trypanosoma*, 3) determine whether land clearing and habitat alterations

associated with subsistence farming within the National Park of American Samoa can influence parasite prevalence, and 4) determine whether parasite prevalence is correlated with seasonal changes in rainfall, temperature and wind speed.

## METHODS

### Mist Netting and Blood Collection

Forest birds were captured with up to 15, 12 or 18 m mist nets (36 mm mesh) between September, 2001 and January, 2011 in or adjacent to plots for subsistence agriculture or in areas with mature native forest on Tutuila and Ta‘u Islands in American Samoa (Figure 1). The few non-native species that have been introduced to American Samoa were either rare or absent from these habitats and were not sampled. Collections on Tutuila were made in low elevation subsistence plots that were adjacent to the main road through Amalau Valley (14° 15′ 19.26″ S, 170° 39′ 30.15″ W, elevation 40 m) and approximately 0.5 km upslope, along a steep, forested ridgeline (Olo Ridge) above the Valley (14° 15′ 29.29″ S, 170° 39′ 26.30″ W, elevation 250 m). Collections on Ta‘u Island were made within a small farm at Faleasao (14° 13′ 11.10″ S, 169° 30′ 14.09″ W, elevation 133 m) and two remote forested sites at Luatele, adjacent to Judd’s Crater (14° 13′ 23.19″ S, 169° 26′ 01.68″ W, elevation 370 m) and Laufuti, adjacent to Laufuti stream in the southern caldera of Mt. Lata (14° 14′ 43.94″ S, 169° 26′ 36.90″ W, elevation 320 m). With the exception of the agricultural site at Faleasao, all others were located within units of the National Park of American Samoa (NPSA) on Tutuila and Ta‘u Islands. Blood collections were made during the warmer, rainy season (November through April) and the cooler, dry season (May through October) (Table 2).

Captured birds were banded with numbered U.S. Geological Survey metal bands, measured, weighed and bled via jugular venipuncture with a heparinized 28-gauge insulin syringe. Up to 100 µl of blood (< 1% of body weight) was collected from each bird. Thin blood smears were made immediately on glass slides, fixed for up to 5 minutes with absolute methanol and air dried. Remaining blood was transferred immediately into tubes with 100 µl of lysis buffer containing 0.1 M tris (hydroxymethyl) aminomethane (Tris), pH 8.0, 0.1 M ethylenediaminetetraacetic acid (EDTA), and 2% sodium dodecyl sulfate (SDS). While in the hand, birds were also examined for crusty and swollen tumor-like lesions on the feet, legs, or head that are characteristic of avian pox.

### Mosquito Sampling

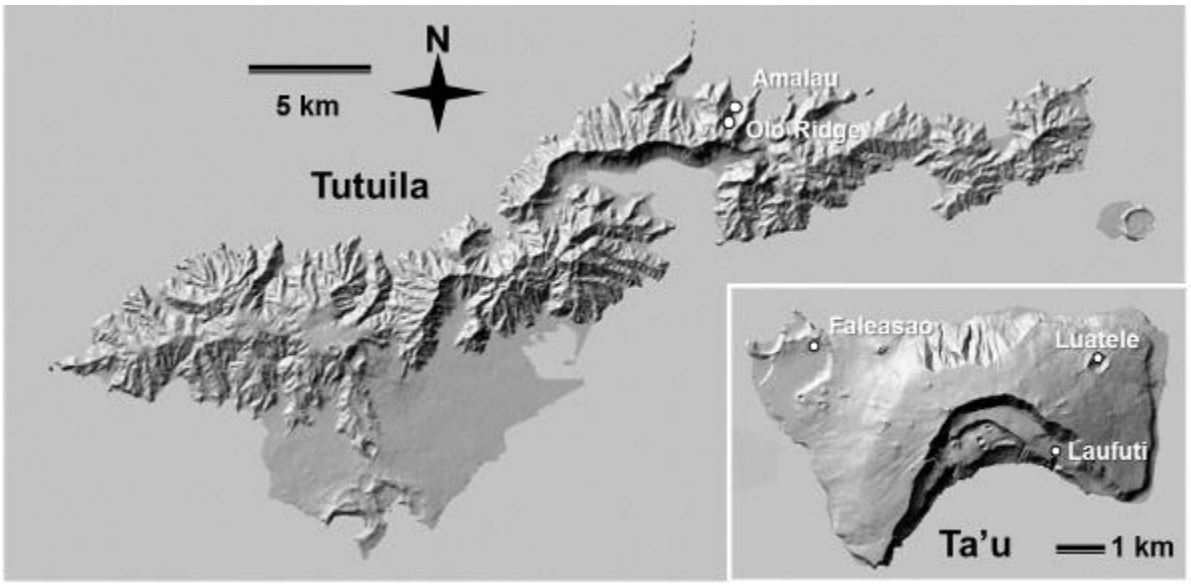
In 2001, five CDC gravid traps (JW Hock Company, Model 1712, Gainesville, FL) baited with a 5-day-old alfalfa and yeast/lactalbumin infusion were used to sample mosquito populations during collecting trips at Amalau Valley. In 2002, four gravid traps and two miniature CDC light traps (JW Hock Company, Model 512, Gainesville, FL) modified without lights and fitted with an ABC CDC adapter and $CO_2$ releasing lid (Clarke Mosquito Control, Model LID1, Roselle, Illinois) and baited with compressed $CO_2$ at 500 ml/min were used to sample mosquitoes at both Amalau Valley and Faleasao. All mosquitoes captured were identified (DAL) and midgut and salivary gland dissections were done within 24 hours of capture with dissecting and compound microscopes to look for oocysts and sporozoites of *Plasmodium* (DAL and CTA).

Table 1. Historic and modern landbirds from Tutuila and the Manu'a Islands of Ofu-Olosega and Ta'u in American Samoa. Limited fossil evidence suggests that this number was once substantially higher and more similar to the diversity of species found in adjacent independent Samoa (Steadman 2006).

| Species | Scientific Name | Islands | Status |
|---|---|---|---|
| **Ardeidae** | | | |
| Pacific reef heron | *Egretta sacra sacra* | Tutuila, Manu'a | I[1], Extant |
| **Anatidae** | | | |
| Pacific black duck | *Anas superciliosa pelewensis* | Tutuila | I, Extant |
| **Phasianidae** | | | |
| Chicken | *Gallus gallus* | Tutuila, Manu'a | A, Extant |
| **Rallidae** | | | |
| Banded rail | *Gallirallus philippensis goodsoni* | Tutuila, Manu'a | I, Extant |
| Spotless crake | *Porzana t. tabuensis* | Manu'a | I, Extant |
| Purple swamphen | *Porphyrio porphyrio samoensis* | Tutuila, Manu'a | I, Extant |
| **Columbidae** | | | |
| Many-colored fruit dove | *Ptilinopus p. perousii* | Tutuila, Manu'a | I, Extant |
| Purple-capped fruit dove | *Ptilinopus porphyraceus fasciatus* | Tutuila, Manu'a | I, Extant |
| Pacific pigeon | *Ducula p. pacifica* | Tutuila, Manu'a | I, Extant |
| Shy ground dove | *Gallicolumba stairi* | Manu'a | I, Extant |
| **Cuculidae** | | | |
| Long-tailed cuckoo | *Eudynamis taitensis* | Tutuila, Manu'a | M[3], Extant |
| **Psittacidae** | | | |
| Blue crowned lorikeet | *Vini australis* | Manu'a | I, Extant |
| **Tytonidae** | | | |
| Barn owl | *Tyto alba delicatula* | Tutuila, Manu'a | A[2], Extant |
| **Apodidae** | | | |
| White-rumped swiftlet | *Collocalia spodiopygia* | Tutuila, Manu'a | I, Extant |
| **Alcedinidae** | | | |
| Collared kingfisher | *Todiramphus chloris manuae/pealei* | Tutuila, Manu'a | I, Extant |
| **Pycnonotidae** | | | |
| Red-vented bulbul | *Pycnonotus cafer* | Tutuila | A, Extant |
| **Monarchidae** | | | |
| Fiji shrikebill | *Clytorhynchus vitiensis* | Manu'a | I, Extant |
| **Sturnidae** | | | |
| Polynesian starling | *Aplonis tabuensis manuae/tutuilae* | Tutuila, Manu'a | I, Extant |
| Samoan starling | *Aplonis atrifusca* | Tutuila, Manu'a | I, Extant |
| Common myna | *Acridotheres fuscus* | Tutuila | A, Extant |
| Jungle myna | *Acridotheres tristis* | Tutuila | A, Extant |
| **Meliphagidae** | | | |
| Cardinal honeyeater | *Myzomela cardinalis* | Tutuila, Manu'a | I, Extant |
| Wattled honeyeater | *Foulehaio c. carunculata* | Tutuila, Manu'a | I, Extant |
| Ma'o | *Gymnomyza samoensis* | Tutuila | I, Extirpated |

[1] Indigenous ,[2] Alien, [3] Migratory

Figure 1. Locations of study sites on Tutuila and Ta'u Islands




More intensive mosquito sampling was done at Amalau Valley, Olo Ridge, Faleasao, Laufuti and Luatele during 2010 and 2011. Because of easy road access, up to six CDC traps baited with compressed $CO_2$ and six BG Sentinel Traps (Biogents AG, Regensburg, Germany) baited with BG lures were deployed at Amalau Valley for as many as six nights per session. Up to three CDC and three BG Sentinel Traps were operated at Olo Ridge, Faleasao, Luatele, and Laufuti from 1–3 nights, depending on weather conditions. Mosquitoes were collected the following morning, sorted to species (MS) and preserved in 100% ethanol (molecular grade) for PCR diagnostics either singly or in pools of up to 10 individuals when capture numbers were high.

**Diagnostics**

*Microscopy*: Slides were stained with 6% Giemsa in 0.01 M sodium phosphate buffer, pH 7.0, for one hour, rinsed with tap water and air dried. Slides were subsequently scanned at 400X (40X objective and 10X eyepiece) to detect hematozoan parasites. We placed a drop of oil and an 18 mm square cover slip at the leading edge of the smear and then examined the entire area of the cover slip at 400X. We estimate that between 30,000 and 50,000 erythrocytes were examined, depending on the thickness of the smear. Parasite identifications (microfilariae, hemococcidians, *Trypanosoma*, *Plasmodium*) were based on morphology. All parasites were photographed with a 100X oil immersion objective for morphological comparison. Pictures were taken with an Olympus BX53 microscope outfitted with a DP73 microscope camera. Measurements were made with Olympus CellSens camera software.

We performed midgut and salivary gland dissections on a subset of mosquitoes that were captured in carbon dioxide-baited CDC traps in May, 2002 at Amalau on Tutuila Island and Faleasao on Ta'u. Tissues from mosquitoes with salivary gland or midgut oocysts, sporozoites, or flagellates were washed from individual slides into vials of lysis buffer for DNA extraction, PCR amplification, and sequencing.

Table 2. Localities, sampling periods and dates for collection of forest bird blood and mosquitoes on Tutuila and Ta‘u Islands

| Locality | Year | Dates | Type |
|---|---|---|---|
| **Tutuila, Amalau Valley** | 2001 | September 5–15 | Blood, Mosquitoes |
| | 2002 | April 24–May 11 | Blood, Mosquitoes |
| | | July 1–3 | Blood |
| | | July 24–August 1 | Blood |
| | | October 31–November 1 | Blood |
| | 2003 | January 7–9 | Blood |
| | | March 17–21 | Blood |
| | | June 17–20 | Blood |
| | | August 1 | Blood |
| | 2007 | July 25–27 | Blood |
| | 2010 | August 16–19 | Blood, Mosquitoes |
| | | December 1–3 | Blood, Mosquitoes |
| | 2011 | March 1–5 | Blood, Mosquitoes |
| **Tutuila, Olo Ridge** | 2010 | April 19–22 | Blood, Mosquitoes |
| | | August 17–19 | Mosquitoes |
| | | November 29–30 | Blood, Mosquitoes |
| | 2011 | March 2–4 | Blood, Mosquitoes |
| **Ta‘u, Faleasao** | 2001 | November 26–30 | Blood |
| | 2002 | April 30–May 3 | Blood, Mosquitoes |
| | 2005 | March 21–24 | Blood |
| | 2006 | September 14–16 | Blood |
| | 2010 | January 26–27 | Mosquitoes |
| | | August 4–12 | Blood, Mosquitoes |
| | 2011 | January 25–28 | Blood, Mosquitoes |
| **Ta‘u, Laufuti** | 2003 | January 21–23 | Blood |
| | 2006 | September 13 | Blood |
| | | November 9 | Blood |
| | | December 9 | Blood |
| | 2010 | August 3–10 | Blood, Mosquitoes |
| | 2011 | January 23–24 | Blood, Mosquitoes |
| **Ta‘u, Luatele** | 2010 | August 9–10 | Blood, Mosquitoes |
| | 2011 | January 19–21 | Blood, Mosquitoes |

*PCR – Blood*: Purified DNA for PCR analysis was extracted from packed blood cells using DNeasy tissue extraction kits (Qiagen Inc., Valencia California) according to manufacturer's protocols but we increased the initial incubation times with Proteinase K to overnight to increase yield of DNA. DNA was recovered from extraction columns with Tris EDTA buffer, measured by spectrophotometry with a Nanodrop spectrophotometer to assess purity and determine DNA concentration, and stored frozen until use in PCR reactions. We screened samples initially for *Plasmodium* and *Haemoproteus* with PCR primers that amplify parasite ribosomal genes (Fallon et al. 2003). The primers were used in a nested protocol with an initial amplification of host and parasite DNA (100 ng/reaction) with primers 292F/631R (Table 3) followed by a second amplification with primers 343F/496R that used 1 µl of a 1:10 dilution of template from the first reaction. PCR reactions with primers 292F/631R were run in 25 µl volumes containing the following components in the reaction mix: 2.0 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.4 µM each primer, and 0.5 units of Promega GoTaq polymerase (Promega North America, Madison, Wisconsin). PCR reactions with primers 343F/496R were run in 25 µl volumes containing the following components in the reaction mix: 2.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 0.5 µM each primer, and 0.25 units of Promega GoTaq polymerase. Cycling conditions for the original flanking primer pair (292F/631R) followed a hot-start, touch-down protocol: 2 min at 94° C, followed by 20 cycles with 1-min denaturation at 94° C, 1-min annealing at 52–42° C, and elongation at 72° C for 1 min and 10 sec. After 20 cycles, a final elongation step followed at 72° C for 3 min. The final assay primer pair (343F and 496R) was run 2 min at 94° C, followed by 35 cycles with 1-min denaturation at 94° C, 1-min annealing at 57° C, and elongation at 72° C for 1 min and 10 sec, with a final elongation step at 72° C for 3 min. PCR products from the second reaction were resolved on 1.5% agarose gels to determine presence or absence of an expected 190 bp band. All PCR reactions were run with a positive control consisting of DNA extracted from a Pekin duckling with an intense experimental infection with *P. relictum* and a negative control that substituted water for DNA.

Blood samples that tested positive with this primer set were reamplified in a nested reaction with flanking primers HAEMNFI/HAEMNR3 and internal primers HAEMF/HAEMR2 with Promega GoTaq polymerase and cycling conditions and reagent concentrations as described by Hellgren et al. (2004). This PCR reaction amplifies a 478 bp portion of the parasite mitochondrial cytochrome b gene which has proven useful for taxonomic and phylogenetic studies of avian species of *Plasmodium* and *Haemoproteus* (Bensch et al. 2009). If we failed to generate a product with the Hellgren primers, we used flanking primers 3760F/4292rw2 as described by Beadell et al. (2004) and the internal primers HAEMF/HAEMR2 as described by Hellgren et al. (2004) to generate a 478 bp product.

We also tested a smaller subset of blood and mosquito samples that were positive by microscopy for *Trypanosoma* with a nested PCR using a set of primers that amplify portions of the trypanosome ribosomal RNA gene (Maslov et al. 1996). The initial reaction used primers S763F/S762R to amply a 2 kb fragment of the gene, followed by a nested reaction using internal primers S823F/S714R to amplify a smaller 1,150 bp fragment for subsequent sequencing. We used internal sequencing primers S825F, and S826R in combination with external primers S823F and S714R to generate overlapping forward and reverse sequences for alignment and construction of a final 1,150 bp contig in Geneious 8 (http://www.geneious.com, Maslov et al. 1996, Kearse et al., 2012) (Table 3). PCR reactions with primers S763/S762 were run in 25 µl volumes containing the following

Table 3. PCR primers used in the study

| Genus | Primer | Sequence 5`– 3` | Reference |
|---|---|---|---|
| ***Plasmodium*** | 292F | CGGTAGATAGGGAACAAACTGC | Fallon et al. 2003 |
| | 631R | GCGAGAAGGGAAGTGTGTTTC | Fallon et al. 2003 |
| | 343F | GCTCACGCATCGCTTCT | Fallon et al. 2003 |
| | 496R | GACCGGTCATTTTCTTTG | Fallon et al. 2003 |
| | HAEMNFI | CATATATTAAGAGAAITATGGAG | Hellgren et al. 2004 |
| | HAEMNR3 | ATAGAAAGATAAGAAATACCATTC | Hellgren et al. 2004 |
| | HAEMF | ATGGTGCTTTCGATATATGCATG | Hellgren et al. 2004 |
| | HAEMR2 | GCATTATCTGGATGTGATAATGGT | Hellgren et al. 2004 |
| | 3760F | GAGTGGATGGTGTTTTAGAT | Beadell et al. 2004 |
| | 4292Rw | TGGAACAATATGTARAGGAGT | Beadell et al. 2004 |
| *Trypanosoma* | S762R | GACTTTTGCTTCCTCTAWTG | Maslov et al. 1996 |
| | S763F | CATATGCTTGTTTCAAGGAC | Maslov et al. 1996 |
| | S823F | CGAAYAACTGCYCTATCAGC | Maslov et al. 1996 |
| | S714R | CGTCAATTTCTTTAAGTTTC | Maslov et al. 1996 |
| | S825F | ACCGTTTCGGCTTTTGTTGG | Maslov et al. 1996 |
| | S826R | CCAACAAAAGCCGAAACGGT | Maslov et al. 1996 |

components in the reaction mix: 1.5 mM $MgCl_2$, 0.25 mM each deoxynucleoside triphosphate (dNTP), 2.0 µM each primer, and 1.0 units of Promega GoTaq polymerase (Promega North America, Madison, Wisconsin). PCR reactions with primers S823/S714 were run in 25 µl volumes containing the following components in the reaction mix: 1.5 mM $MgCl_2$, 0.2 mM each deoxynucleoside triphosphate, 1.25 µM each primer, and 1.0 units of Promega GoTaq polymerase. Cycling conditions for primers S763/S762 were an initial denaturation at 95° C for 5 min followed by five cycles at 95° C for 1 min, 45° C for 30 sec, 65° C for 1 min and 35 cycles at 95° C for 1 min, 50° C for 30 sec, 72° C for 1 min and a final extension at 65° C for 10 min. Cycling conditions for primers S823/S714 were an initial denaturation at 95° C for 3 min followed by 35 cycles at 95° C for 30 sec, 57° C for 1 min, 72° C for 1 min and a final extension at 72° C for 7 min. PCR products from the second reaction were resolved on 1.5% agarose gels to determine presence or absence of an expected 1,150 bp band. All PCR reactions were run with a positive control consisting of DNA extracted from a bird that was positive for *Trypanosoma* based on microscopy and a negative control that substituted water for DNA.

*PCR – Mosquitoes*: Ethanol–preserved mosquitoes were dissected prior to extraction either singly or in pools of up to 10 individuals of the same species to separate head and thorax from the abdomen. Dissecting tools were dipped in 10% bleach and rinsed with 70% ethanol after each pool was handled. Single or pooled heads and thoraxes were analyzed separately from corresponding single or pooled abdomens so that salivary gland sporozoites (thorax) could be distinguished from developing oocysts or parasites present within partially or completely digested blood meals (abdomen). Up to 500 individuals of each species were tested. Pooled or individual abdomens and thoraxes were ground in 180 µl of phosphate-buffered saline (PBS) with an electric homogenizer (Fisher Scientific, catalog #12-141-361) and a disposable plastic pestle (Fisher Scientific, catalog #12-141-364) and extracted with DNeasy tissue extraction kits according to the manufacturer's protocol for soft bodied insects. Extracted DNA was screened by PCR for *Plasmodium* with either HAEMNFI/HAEMNR3 or 3760F/4292rw2 flanking primers and

HAEMF/HAEMR2 nested primers as described earlier for blood samples. Mosquitoes that were positive for *Trypanosoma* by microscopy were screened by PCR for *Trypanosoma* as described earlier.

*Sequencing:* All PCR products that were generated for sequencing were cleaned and purified with ExoSAP-IT (Affymetrix Inc, Cleveland, OH) or Qiagen MiniElute PCR Purification Kits and sequenced in both directions on an ABI 3730XL capillary DNA sequencer (Sequetech, Mountain View, California) or an ABI 3500 Genetic Analyzer (UH-Hilo Core Genetics Lab, Hilo, HI). All sequences were aligned and proofed using Geneious R7 or R8.

**Climate Data**

Daily temperature, rainfall and windspeed data from the National Oceanic and Atmospheric Administration (NOAA) weather station at Pago Pago International Airport on Tutuila were used to compare mean seasonal rainfall totals, windspeed, and maximum/minimum temperatures for the summer (November–April) and winter (May–October) seasons. Comparable weather data is not available from Ta'u Island.

**Data Analysis**

*Prevalence – Birds*: We used binary logistic regression in the program Systat Version 11 (2004) to test for associations between infection status and the independent covariates of Island, Land Use (agriculture or native forest), Island*Land Use interactions, and Season based on temperature and rainfall differences between the warmer, wetter months between November and April (summer) and the cooler, dryer months between May and October (winter). To determine the effects of time on prevalence, we used a subset of data that included collections made across multiple years at Amalau (2001–2011), Faleasao (2001–2011) and Laufuti (2003–2011) and tested for effects of Calendar Year, Season*Calendar Year, and Island*Calendar Year interactions. Logistic models were fitted using forward stepwise regression with entry $P$ values of $P < 0.15$ and retention $P$ values of $P < 0.05$.

We used Pearson Chi Square (Systat Version 11) for individual comparisons of prevalence by parasite type (*Plasmodium*, *Trypanosoma*, microfilaria, *Isospora*), parasite lineage, island, land use, and season. Level of significance for all statistical comparisons was $P < 0.05$.

*Prevalence – Mosquitoes*: We estimated minimum prevalence in the vector population by assuming that pooled abdomens or thoraxes from individual species of mosquitoes that were positive by PCR contained at least one infected mosquito.

*Phylogenetics*: Forward and reverse sequences from samples that were positive for *Plasmodium* were aligned in Geneious 8, trimmed to a 479 bp reference sequence (*P. relictum* SGS1, AF495571) and edited to check for ambiguous bases. We constructed a phylogenetic tree using the MrBayes plugin for Geneious R8 to determine taxonomic relationships among lineages of *Plasmodium* that we identified in American Samoa with comparable sequence data from known morphospecies of *Plasmodium* from MalAvi (http://mbio-serv2.mbioekol.lu.se/Malavi/) and GenBank (Table 4). An alignment of known species and unique sequences identified in this study was trimmed to the shortest sequence (468 bp) and tested by MrModeltest version 2.3 (Posada and Crandall, 1998) to determine an appropriate model of sequence evolution for use in the Bayesian phylogenetic analysis. Four species of avian *Leucocytozoon* (*L. buteonis*, *L. mathisi*, *L. sabrazesi* and *L. quynzae*) were selected as outgroups. For the Bayesian analysis we ran 10,000,000 generations with a sampling frequency of every 1,000 generations using a

Markov chain Monte Carlo (MCMC) algorithm and a General Time Reversible Model (GRT + I + G). The first 25% of trees were discarded as burn-in.

A phylogenetic tree that compared 18S ribosomal RNA sequence data for avian *Trypanosoma* was generated in a similar manner with the MrBayes plugin for Geneious R8. Sequences we obtained from American Samoa were aligned in Geneious 8, trimmed and edited to check for ambiguous bases. American Samoan sequences were aligned with 18S ribosomal RNA sequences reported by Zídková et al. (2012) from European birds (Appendix 1). The sequences were trimmed to the shortest sequence (1239 bp) and tested by MrModeltest version 2.3 (Posada and Crandall 1998) to determine the most appropriate model of sequence evolution. For the Bayesian analysis we ran 10,000,000 generations with a sampling frequency of every 1,000 generations using the Markov chain Monte Carlo (MCMC) algorithm and a General Time Reversible Model (GRT + I + G). We used four species of mammalia *Trypanosoma* as an outgroup.

## RESULTS

### Prevalence

*Host Species*: Infections with *Plasmodium*, *Trypanosoma*, filarial worms and haemococcidians (*Isospora*) were most common in Polynesian starlings (46%, 21/46), Wattled honeyeaters (37%, 148/404), Samoan starlings (36%, 56/155), Cardinal honeyeaters (17%, (1/6), and White-rumped swiftlets (20%, 4/20) (Table 5, Figure 2). Ninety-five percent (225/238) of these infections were detected in Samoan starlings, Polynesian starlings and Wattled honeyeaters alone. These three species made up more than 75% of all captures and dominated the forest bird community at all four study sites. Among infections, *Plasmodium* was most common in Polynesian starlings (24%, 11/46), Wattled honeyeaters (19%, 78/404), and Samoan starlings (18%, 28/155); *Trypanosoma* was most common in Samoan starlings (11%, 17/149), Fiji shrikebills (7%, 1/15), and Polynesian starlings (5%, 2/43); filarial worms were most common in Wattled honeyeaters (17%, 64/385), Polynesian starlings (14%, 6/43) and Samoan starlings (7%, 10/149); haemococcidians were most common in White-rumped swiftlets (5%, 1/19), Polynesian starlings (5%, 2/43), and Blue-crowned lorikeets (4%, 1/26) (Figure 2).

By contrast, infections were relatively rare, present in a single captured individual, or not detected at all in the remaining forest bird community. Parasites were detected in only 8% (3/40) of Purple-capped fruit doves (*Plasmodium* and filarial worms), 5% (1/19) of Fiji shrikebills (*Trypanosoma*), 4% (1/26) of Blue-crowned lorikeets (*Isospora*), 3% (2/75) of Collared kingfishers (*Plasmodium* and filarial worms), and 100% (1/1) in a single Long-tailed cuckoo (*Plasmodium*). We did not detect parasites in the remaining species that were captured. These included a single Pacific reef heron, a single Purple swamphen, a single Many-colored fruit dove, a single Barn owl, a single Red-vented bulbul and three Pacific pigeons (Table 1).

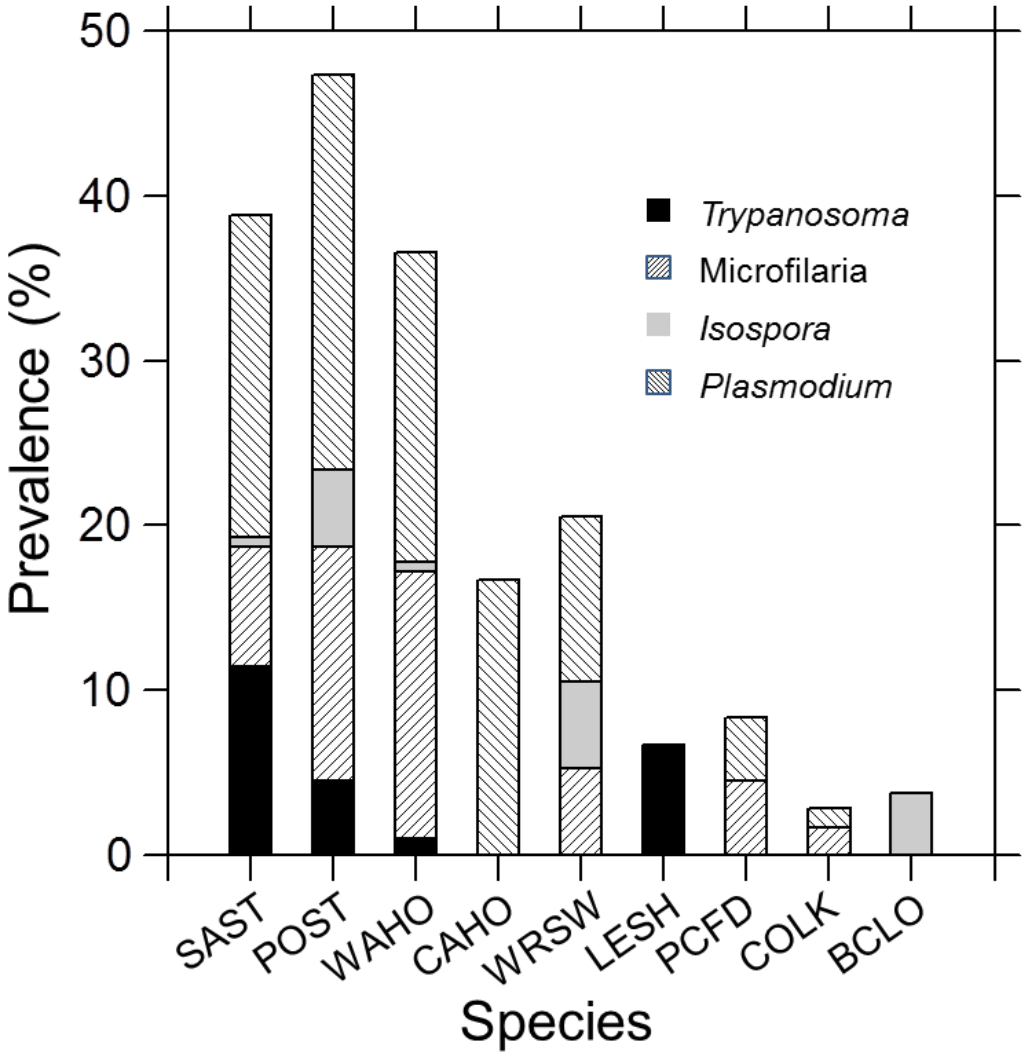
Table 4. Pairwise percent differences in nucleotide sequences among lineages found in forest birds (033, 3641, 8825) and mosquitoes (9080, 9142) from American Samoa and closely related lineages of avian *Plasmodium* from MalAvi that have not been assigned to a morphological species (P_ORW1, P_SCEDEN01, P_AEGTIP01, P_COQUI01, P_PITINC01, P_UPUPA01, P_XANFLA04). Note absence of any differences between 3641 and P_SCEDEN01 (0%), 033 and P_ORW1 (0%), and a minor difference (0.22%) between 8825, 033, and P_ORWI that resulted from a single SNP at position 212. Lineages found in mosquitoes (9080, 9142) were most similar to GALLUS02 (not shown), a lineage from Asian birds that has been identified as *P. juxtanucleare*.

| Lineage | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **033_A_tabuensis_Tau** | **1** | | 5.79 | 0.22 | 6.46 | 6.68 | 6.01 | 0.22 | 0 | 0.45 | 5.79 | 0.84 | 5.57 |
| **3641_F_carunculatus_Tutuila** | **2** | 5.79 | | 5.79 | 8.46 | 8.69 | 0.67 | 6.01 | 5.79 | 5.35 | 0 | 5.51 | 0.22 |
| **8825_A_atrifusca_Tau** | **3** | 0.22 | 5.79 | | 6.46 | 6.68 | 6.01 | 0.45 | 0.22 | 0.67 | 5.79 | 1.06 | 5.57 |
| **9080_C_sitiens_Tutuila** | **4** | 6.46 | 8.46 | 6.46 | | 0.22 | 8.69 | 6.68 | 6.46 | 6.68 | 8.46 | 6.63 | 8.24 |
| **9142_C_sitiens_Tutuila** | **5** | 6.68 | 8.69 | 6.68 | 0.22 | | 8.91 | 6.9 | 6.68 | 6.9 | 8.69 | 6.85 | 8.46 |
| **P_AEGTIP01** | **6** | 6.01 | 0.67 | 6.01 | 8.69 | 8.91 | | 6.24 | 6.01 | 5.57 | 0.67 | 5.73 | 0.89 |
| **P_COQUI01** | **7** | 0.22 | 6.01 | 0.45 | 6.68 | 6.9 | 6.24 | | 0.22 | 0.67 | 6.01 | 1.06 | 5.79 |
| **P_ORW1** | **8** | 0 | 5.79 | 0.22 | 6.46 | 6.68 | 6.01 | 0.22 | | 0.45 | 5.79 | 0.84 | 5.57 |
| **P_PITINC01** | **9** | 0.45 | 5.35 | 0.67 | 6.68 | 6.9 | 5.57 | 0.67 | 0.45 | | 5.35 | 1.28 | 5.12 |
| **P_SCEDEN01** | **10** | 5.79 | 0 | 5.79 | 8.46 | 8.69 | 0.67 | 6.01 | 5.79 | 5.35 | | 5.51 | 0.22 |
| **P_UPUPA01** | **11** | 0.84 | 5.51 | 1.06 | 6.63 | 6.85 | 5.73 | 1.06 | 0.84 | 1.28 | 5.51 | | 5.29 |
| **P_XANFLA04** | **12** | 5.57 | 0.22 | 5.57 | 8.24 | 8.46 | 0.89 | 5.79 | 5.57 | 5.12 | 0.22 | 5.29 | |

Table 5. Overall prevalence (%) of *Plasmodium*, *Trypanosoma*, microfilaria, and *Isospora* by host species at five study sites on Tutuila and Ta'u Islands. Blood samples were collected between 2001 and 2011. Prevalence of *Plasmodium* is based on the initial diagnostic screen with PCR primers 292F/631R and 343F/496R. Prevalence of *Trypanosoma*, microfilaria and *Atoxoplasma* is based on microscopic examination of blood smears.

| Species | *Plasmodium* | *Trypanosoma* | Microfilaria | *Isospora* |
|---|---|---|---|---|
| **Ardeidae** | | | | |
| Pacific reef heron | 0 (0/1) | 0 (0/1) | 0 (0/1) | 0 (0/1) |
| **Rallidae** | | | | |
| Purple swamphen | 0 (0/1) | 0 (0/1) | 0 (0/1) | 0 (0/1) |
| **Columbidae** | | | | |
| Many-colored fruit dove | 0 (0/1) | 0 (0/1) | 0 (0/1) | 0 (0/1) |
| Purple-capped fruit dove | 5.0 (2/40) | 0 (0/40) | 2.5 (1/40) | 0 (0/56) |
| Pacific pigeon | 0 (0/3) | 0 (0/3) | 0 (0/3) | 0 (0/3) |
| **Psittacidae** | | | | |
| Blue-crowned lorikeet | 0 (0/26) | 0 (0/26) | 0 (0/26) | 3.8 (1/26) |
| **Cuculidae** | | | | |
| Long-tailed cuckoo | 100 (1/1) | 0 (0/1) | 0 (0/1) | 0 (0/1) |
| **Tytonidae** | | | | |
| Barn owl | 0 (0/1) | 0 (0/1) | 0 (0/1) | 0 (0/1) |
| **Apodidae** | | | | |
| White-rumped swiftlet | 10.0 (2/20) | 0 (0/19) | 5.3 (1/19) | 5.3 (1/19) |
| **Alcedinidae** | | | | |
| Collared kingfisher | 1.3 (1/75) | 0 (0/74) | 1.4 (1/74) | 0 (0/74) |
| **Pycnonotidae** | | | | |
| Red-vented bulbul | 0 (0/1) | 0 (0/1) | 0 (0/1) | 0 (0/1) |
| **Monarchinae** | | | | |
| Fiji shrikebill | 0 (0/19) | 6.7 (1/15) | 0 (0/15) | 0 (0/15) |
| **Sturnidae** | | | | |
| Polynesian starling | 23.9 (11/46) | 4.7 (2/43) | 14.0 (6/43) | 4.7 (2/43) |
| Samoan starling | 19.3 (28/155) | 11.4 (17/149) | 6.7 (10/149) | 0.7 (1/149) |
| **Meliphagidae** | | | | |
| Cardinal honeyeater | 14.3 (1/6) | 0 (0/6) | 0 (0/6) | 0 (0/6) |
| Wattled honeyeater | 21.6 (78/404) | 1.0 (4/385) | 16.6 (64/385) | 0.5 (2/385) |
| **Total** | 15.5 (124/800) | 3.1 (24/766) | 10.7 (82/766) | 0.9 (7/766) |

Figure 2. Overall prevalence (%) of *Plasmodium*, *Trypanosoma*, microfilaria, and *Isospora* by host species at five study sites on Tutuila and Ta'u Islands. Samoan starling (SAST), Polynesian starling (POST), Wattled honeyeater (WAHO), Cardinal honeyeater (CAHO), White-rumped swiftlet (WRSW), Fiji shrikebill (LESH), Purple-capped fruit dove (PCFD), Collared kingfisher (COLK), and Blue-crowned lorikeet (BCLO).



*Island:* Combined prevalence of infection with all four parasites was significantly higher on Ta'u (44%, 178/405) than Tutuila (15%, 60/395) ($X^2$ = 79.146, df = 1, $P$ < 0.0001). This difference was a result of a significantly higher prevalence of *Plasmodium* (21.5%, 87/405, $X^2$ = 22.407, df = 1, $P$ < 0.0001) and microfilaria (20.4%, 77/378, $X^2$ = 70.223, df = 1, $P$ < 0.0001) on Ta'u relative to Tutuila where prevalence of *Plasmodium* and microfilaria was 9.4% (37/395) and 1.6% (6/382), respectively. Prevalence of *Trypanosoma* did not differ significantly between Tutuila (3.9%, 15/388) and Ta'u (2.4%, 9/378) ($X^2$ = 1.391, df = 1, $P$ = 0.238). Similarly, prevalence of *Isospora* did not differ significantly between the two islands (Tutuila, 0.5%, 2/388; Ta'u, 1.3%, 5/378) ($X^2$ = 1.378, df = 1, $P$ = 0.240) (Figure 3).

*Habitat:* Combined prevalence of infection with all four parasites was significantly higher at study sites with subsistence agriculture (Amalau, Faleasao; 37.5%, 221/590) than those in more pristine forests in more remote areas of NPSA (Olo Ridge, Luatele, Laufuti; 7.6%, 16/210) ($X^2$ = 66.139, df = 1, $P$ < 0.0001) in spite of similar host communities at each site (Figure 4). When prevalence was broken down by parasite, significant differences between agricultural and undeveloped native forest were evident for *Plasmodium* (Park = 2.9%, 6/210; Ag = 20.0%, 118/590; $X^2$ = 34.75, df = 1, $P$ < 0.0001) and filarial worms (Park = 2.1%, 4/187; Ag = 13.5%, 78/579; $X^2$ = 18.99, df = 1, $P$ < 0.0001), but not for *Trypanosoma* (Park = 1.6%, 3/187; Ag = 3.6%, 21/579; $X^2$ = 1.905, df = 1, $P$ = 0.167) or *Isospora* (Park = 0.7%, 4/579; Ag = 1.6%, 3/187; $X^2$ = 1.302, df = 1, $P$ = 0.254). Most of the significant difference for infection with *Plasmodium* and microfilaria was accounted by their relatively high prevalence of infection on agricultural land at Faleasao on Ta'u Island (*Plasmodium*: 40.5%, 85/210; microfilaria: 29.8%, 76/255). By contrast, prevalence of *Plasmodium* and microfilaria in more remote forests of Laufuti and Luatele on Ta'u was only 1.4% (2/147) for *Plasmodium* and 1.6% (2/123) for microfilaria. Combined prevalence of infection was also higher on agricultural land on Tutuila (15.4%, 51/332) relative to nearby park lands on Olo Ridge (7.6%, 16/210), but this difference was not statistically significant ($X^2$ = 0.296, df = 1, $P$ = 0.587).

*Season:* We observed little seasonal and yearly variability in mean minimum and maximum temperature over the course of the study (Figure 5). Mean minimum daily temperatures ranged from 24–25 $^{o}$C in both winter and summer, while mean maximum daily temperatures were 1–2 $^{o}$C higher during the summer. By contrast, mean daily windspeeds were lower during the summer months. Mean rainfall totals were more variable from year to year and generally higher during the summer months.

Combined prevalence of *Plasmodium*, *Trypanosoma*, microfilaria, and *Isospora* was significantly higher during the warm, wet summer season between November and April (23.8%, 111/466), than cooler, dryer winter months between May and October (18.9%, 63/334) ($X^2$ = 4.780, df = 1, $P$ = 0.029), but this difference was due to a significantly higher seasonal prevalence of microfiliaria during the summer months (summer, 13.3%, 58/435; winter 8.5%, 28/328) ($X^2$ = 4.302, df = 1, $P$ = 0.038). While combined prevalence was higher in the summer relative to winter, it was not significantly different for *Plasmodium* (summer, 16.3%, 76/466; winter, 14.4%, 48/334; $X^2$ = 2.171, df = 1, $P$ = 0.141) or *Trypanosoma* (summer, 3.9%, 17/435; winter 2.1%, 7/328; $X^2$ = 1.931, df = 1, $P$ = 0.165). Prevalence of *Isospora* was slightly lower in the summer, but did not differ significantly from winter (summer, 0.7%, 3/435; winter 1.2%, 4/328; $X^2$ = 0.578, df = 1, $P$ = 0.447).

Figure 3. Prevalence (%) of *Plasmodium*, *Trypanosoma*, microfilaria, and *Isospora* by island.

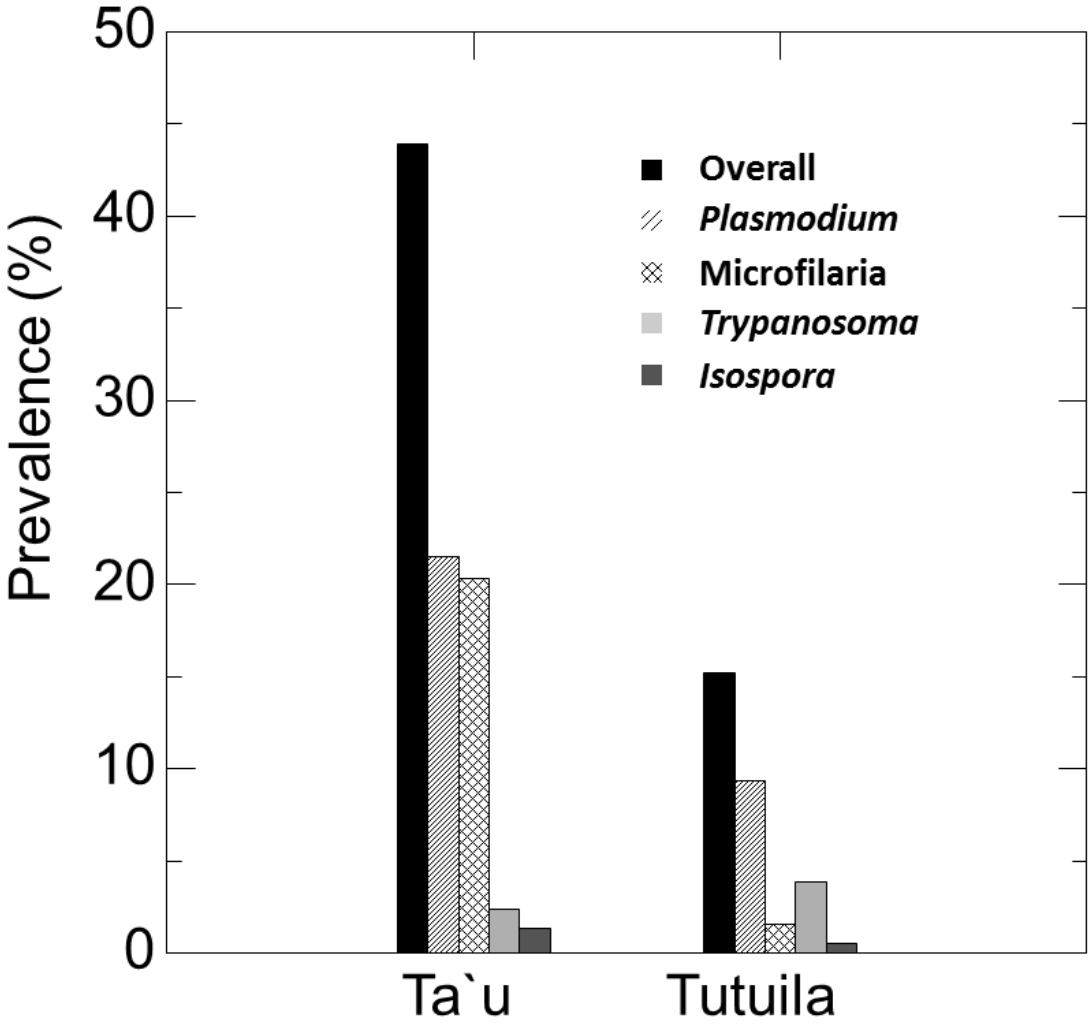


Figure 4. Overall prevalence (%) of *Plasmodium*, *Trypanosoma*, microfilaria, and *Isospora* on Tutuila and Ta'u Islands by land use. Agricultural (AG) sites include Amalau on Tutuila and Faleasao on Ta'u Islands. Undeveloped native forest sites (PARK) include Olo Ridge on Tutuila and Luatele and Laufuti on Ta'u Islands.

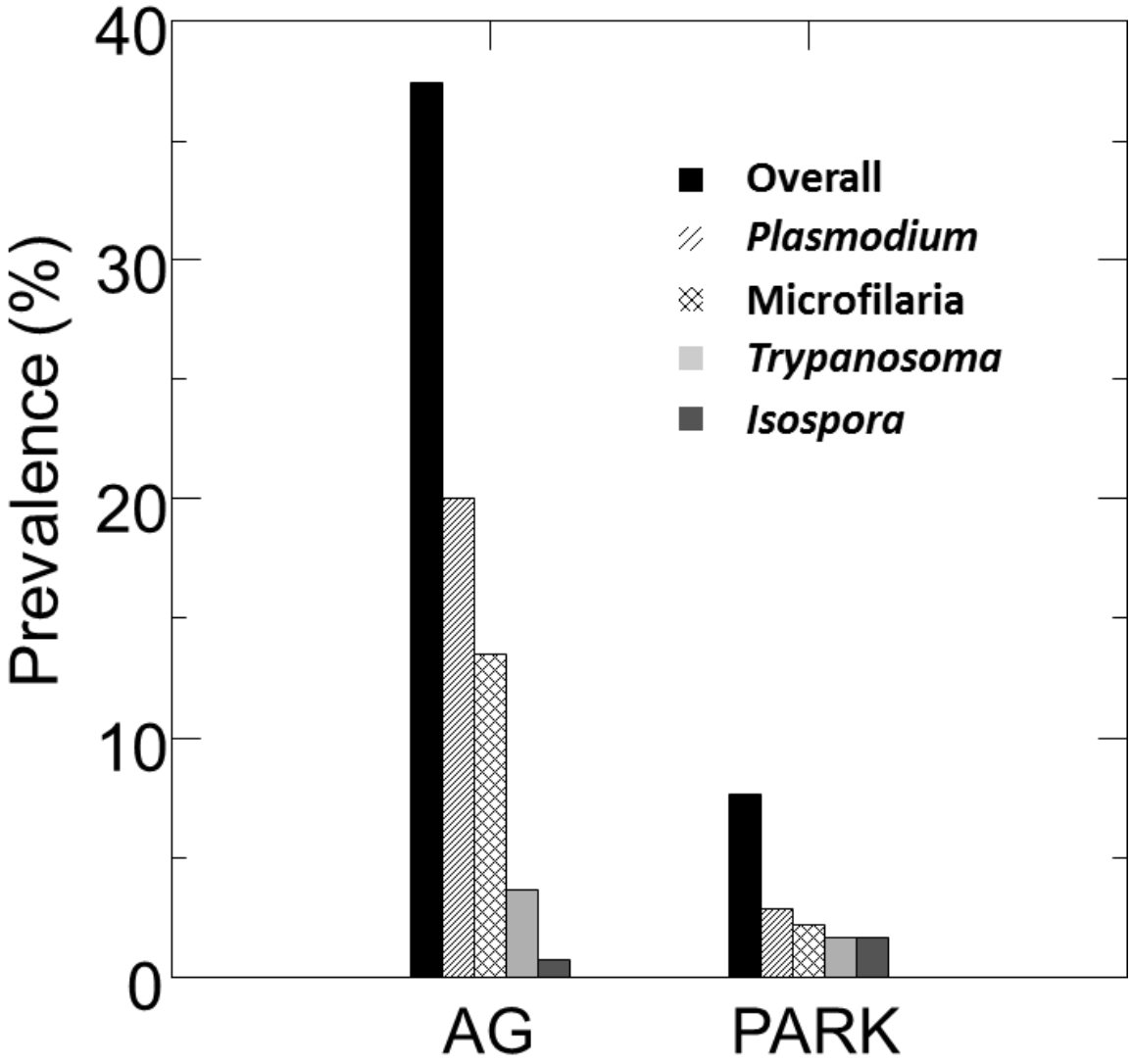
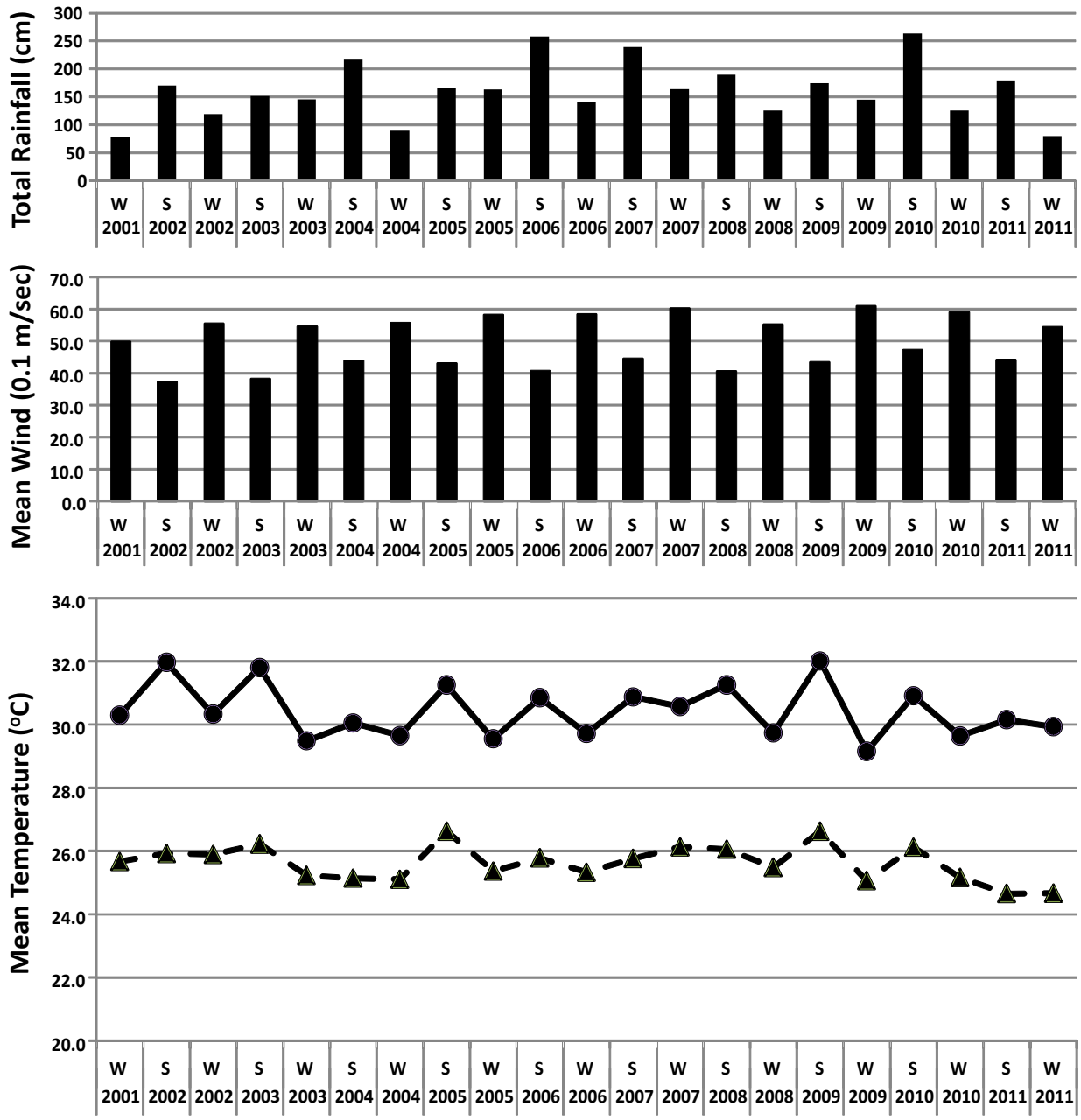


Figure 5. Total Rainfall, mean daily wind speed, and mean daily minimum (▲) and maximum (●) temperatures for winter (May–October) and summer (November–April) seasons at Pago Pago International Airport.



*Land Use and Season:* Significant effects on combined infection status were associated with Island ($P = 0.002$) and Island*Land Use interaction ($P < 0.0001$). We did not find any significant association between infection status and either Land Use ($P = 0.558$) or Season ($P = 0.391$).

*Year:* Since sampling effort at all five study sites was not equal over the course of the 10 year study, we compared yearly differences in prevalence at the three sites that were sampled repeatedly over the longest time period—Amalau on Tutuila and Faleasao and Laufuti on Ta'u (Table 2, Figure 5). Significant effects on combined infection status were associated with Site ($P < 0.0001$). We did not find any significant Year ($p = 0.161$) or Year*Site interactions ($p = 0.431$).

**Taxonomy and Morphology**

*Plasmodium:* We were able to obtain sequence data from 84% (104/124) of samples that were positive for *Plasmodium* by our initial screen with nested PCR primers that amplify parasite ribosomal genes. We identified two previously reported lineages of *Plasmodium*, SCENDEN01 and ORW1, that have been described from a variety of avian hosts in Europe, Australasia and Oceania but have not been linked to any morphologically defined species. With the exception of a single infection from a Samoan starling on Ta'u, both lineages were 100% identical to sequences deposited in MalAvi (http://mbio-serv2.mbioekol.lu.se/Malavi/) from species of Passeriformes (SCENDEN01, ORW1), Falconiformes (ORW1), Coraciiformes (ORW1), and Piciformes (ORW1) in Australia (SCEDEN01) and Europe, Australasia, and Oceania (ORW1). The single variant of ORW1 that we identified had a single G/C single nucleotide polymorphism (SNP) at position 212 of the 478 bp fragment of the cytochrome b gene.

SCENDEN01 was the most common lineage of *Plasmodium* in American Samoa and comprised approximately 80% (83/104) of the infections that we were able to type by sequencing. The relative distribution of ORW1 and SCENDEN01 was similar on both Tutuila and Ta'u Islands with SCENDEN01 making up approximately 79% (19/24) of infections on Tutuila and 80% (64/80) of infections on Ta'u. SCEDEN01 was most common in Wattled honeyeaters (78%, 65/83), followed by Samoan starlings (12%, 10/83), Polynesian starlings (7%, 6/83), Cardinal honeyeaters (1%, 1/83) and White-rumped swiftlets (1%, 1/83). By contrast, ORW1 was most common in Samoan starlings (76%, 16/21), followed by Polynesian starlings (19%, 4/21) and Wattled honeyeaters (5%, 1/21).

Prevalence of *Plasmodium* (SCEDEN01 and ORW1) by microscopy was low relative to PCR diagnostics and we detected erythrocytic stages of the parasite on Giemsa-stained blood smears in only 8.9% (11/124) of the samples that were positive by our initial screening with nested PCR primers that amplified parasite ribosomal RNA genes. With the exception of a positive Polynesian starling from Ta'u that was infected with lineage ORW1 (Figure 6), parasitemia was extremely low and normally less than 1% of circulating erythrocytes. This made description of morphological features of the parasites difficult and we were forced to use parasites from multiple avian hosts to describe features of lineage SCEDEN01 (Figure 7). Both lineages had morphological features that are consistent with species of *Plasmodium* from the subgenus Giovannolaia—meronts that are greater in size than the nuclei of red blood cells and gametocytes that partially or completely surrounded the host cell nucleus. Lineage ORW1 could be distinguished from lineage SCEDEN01 by presence of elongate cytoplasmic processes that were either amoeboid in form or sharply pointed on immature and mature meronts and immature gametocytes (Figures 7 and 8). In both cases, numbers of parasites that we found in

infections with SCENDEN01 were low, making it difficult to identify key diagnostic features in trophozoites and gametocytes that can be used to reliably distinguish this lineage from ORW1.

Figure 6. Overall prevalence of *Plasmodium*, *Trypanosoma*, microfilaria and *Isospora* by year at Amalau on Tutuila Island and Faleasao and Laufuti on Ta'u Island where long-term sampling was done. Field collections at Olo Ridge and Luatele are not included.

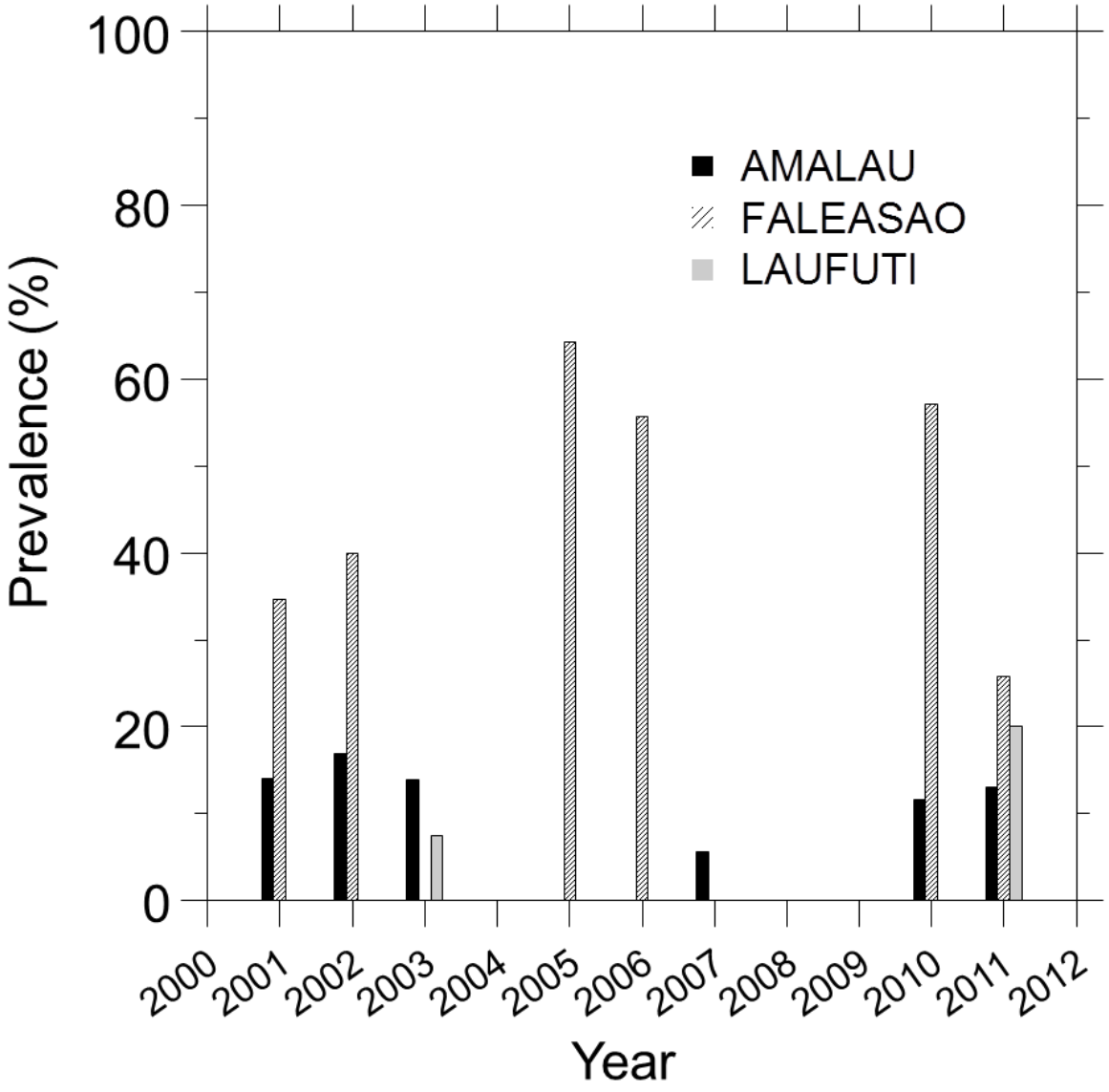


Figure 7. Asexual (a–i) and sexual (j–l) stages of *Plasmodium* lineage SCEDEN01 from a Wattled honeyeater (k, l) and a Cardinal honeyeater (a–j) captured on Tutuila Island. Immature (d–f) and mature meronts (g–i) partially or completely surround the host nucleus and cause some lateral displacement. Immature (j) and mature (k, l) gametocytes partially or completely surround the host cell nucleus. Microgametocytes were not clearly distinguishable on this Giemsa-stained blood smear.

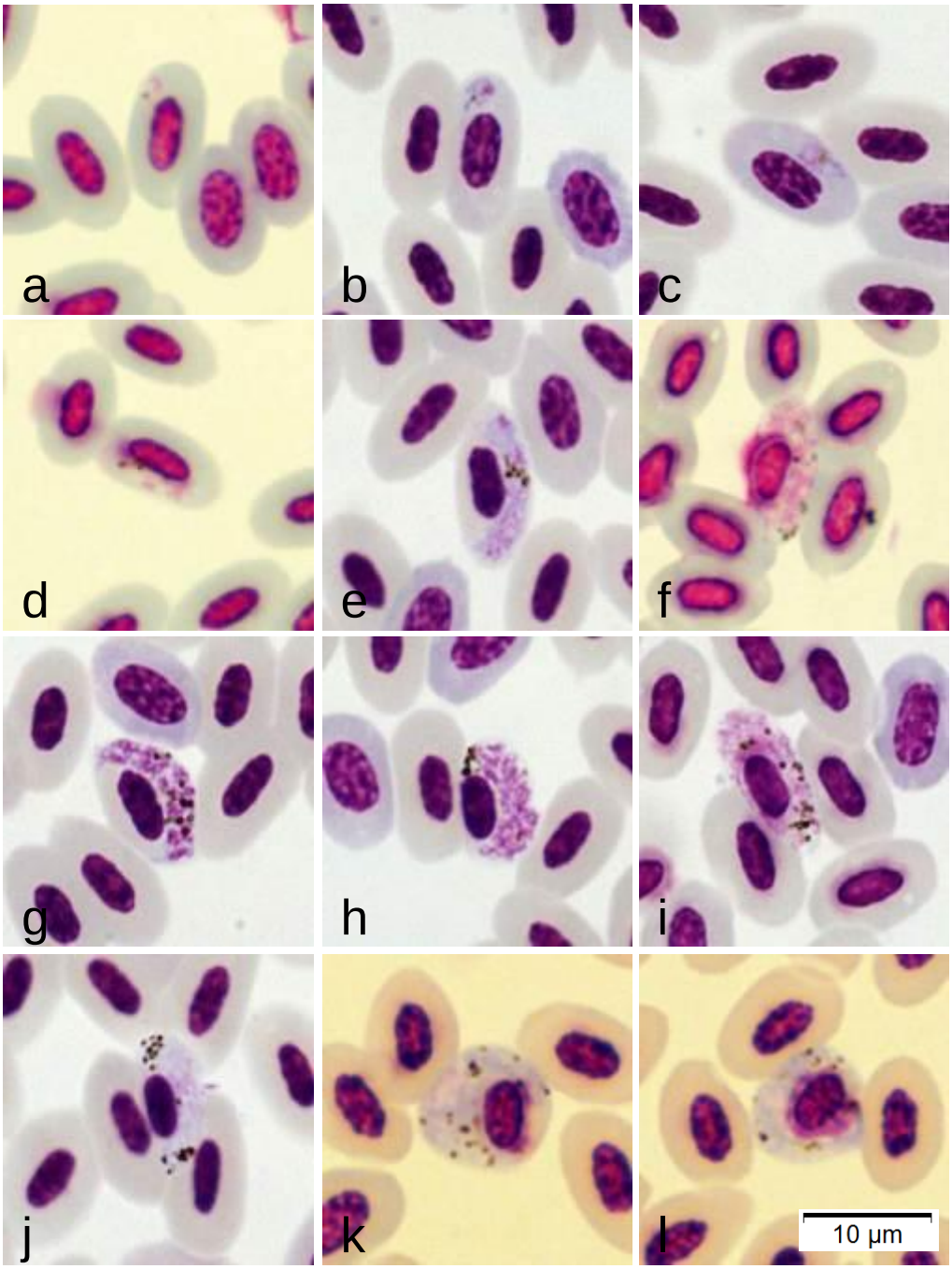
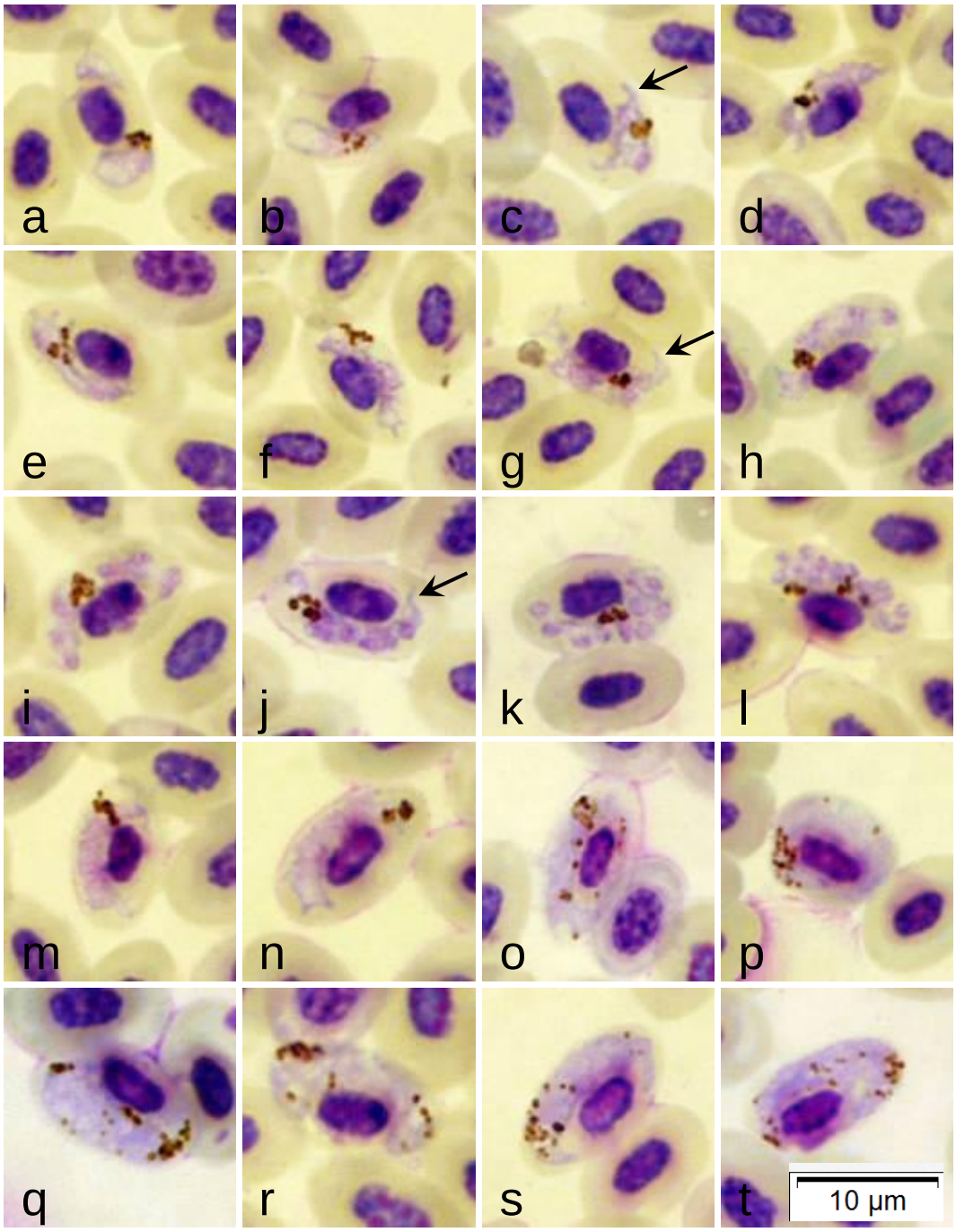


Figure 8. Asexual (a–l) and sexual (m–t) stages of *Plasmodium* lineage ORW1 from a Polynesian starling captured on Ta'u Island. Note elongate and pointed cytoplasmic extensions on immature meronts (c–j) (arrows). Mature schizonts (k, l) partially surround the host cell nucleus and cause minor lateral displacement. Note cytoplasmic extensions on immature gametocytes (m, n) that partially surround host cell nucleus. Mature macrogametocytes (o–t) partially or completely surround the host cell nucleus. Microgametocytes were not clearly distinguishable on this Giemsa-stained blood smear.



In spite of morphological similarities between ORW1 and SCEDEN01, the parasites were taxonomically unique from each other and formed highly supported independent clades from described species of *Plasmodium* based on a Bayesian analysis of the 478 bp barcoding region of the parasite cytochrome b gene (Figure 9). Pairwise percent differences in nucleotide sequences exceeded 5% for all comparisons with known species of *Plasmodium*. When comparisons were made among American Samoan lineages and their closest relatives in the MalAvi database, differences ranged from 5%–6% for comparisons between SCEDEN01 and ORW1 and < 2% for comparisons among MalAvi lineages that were most closely related to either SCEDEN01 or ORW1 (Table 4).

*Trypanosoma*: We observed a single morphological type of *Trypanosoma* in forest birds from American Samoa. Circulating trypomastigotes were large in size (43–58 µm in length, excluding the free flagellum) and had prominent pellicular striations, finely pointed posterior ends, and a kinetoplast that was located approximately 1/3 of the body length from the posterior end (Table 6, Figure 10).

Phylogenetic analysis of 18s ribosomal sequences placed all of the American Samoa trypanosomes within the *T. culicavium*/*T. corvi* clade of avian trypanosomes - one of the three well supported clades of avian trypanosomes reported by Zídková et al. (2012) (Figure 11). In spite of this similarity, most morphological measurements did not overlap with those of either T. culicavium or T. corvi (Table 6; PA, FF, KN, PK, PN, PK/PA, PN/KN, PN/NA, PN/PA).

*Microfilaria: Microfilaria* were similar in morphology among all avian hosts that were positive by microscopy. The nematodes measured from 152–270 µm in length ($X = 223 \pm 20.9$, $n = 34$), had blunt anterior ends and pointed posterior ends, and were surrounded by a sheath that frequently extended beyond both anterior and posterior ends of microfilariae (Figure 12).

*Haemococcidians:* Detections of *Isospora* (synonym = *Atoxoplasma*) by microscopy were rare (1.2%, 9/766) among slides that we examined. Parasites were observed within the cytoplasm of circulating lymphocytes as spherical, pale staining bodies with finely granular cytoplasm and a pale pink nucleus that was not always evident (Figure 13). Host lymphocytes had deeply staining magenta nuclei and deep blue cytoplasm. Host cell nuclei frequently appeared pyknotic and degenerative and eventually formed vacuoles that were adjacent to or surrounding developing parasites (Figure 13). The cytoplasm of infected lymphocytes was often darkly stained and surrounded an often poorly stained trophozoite.

**Vectors**

We collected mosquitoes at all five study sites, but limited diagnostic screening by PCR to the three sites with highest prevalence of infection in forest birds—Amalau, Faleasao, and Olo Ridge. We amplified SCEDEN01 and two closely related *Plasmodium* lineages from both pooled and individual mosquitoes. These two lineages were not detected in forest birds and differed from each other by only 1 base pair. They were most similar to GALLUS02 - a lineage that has been identified in previous studies as *P. juxtanucleare*, a species that is common in Galliformes, including domestic chickens (*Gallus gallus*) from Asia, South America and Africa.

Figure 9. Bayesian inference phylogenetic reconstruction based on 468 nucleotides of the cytochrome b gene of avian *Plasmodium*. The tree includes sequence data from known morphological species of avian *Plasmodium* plus the most closely related lineages to SCEDEN01 (XANFLA04, AEGTIP01) and ORW1 (UPUPA01, PITINC01, COQUI01) that are currently available in MalAvi (http://mbio-serv2.mbioekol.lu.se/Malavi/). Four lineages of *Leucocytozoon* are included as an outgroup.

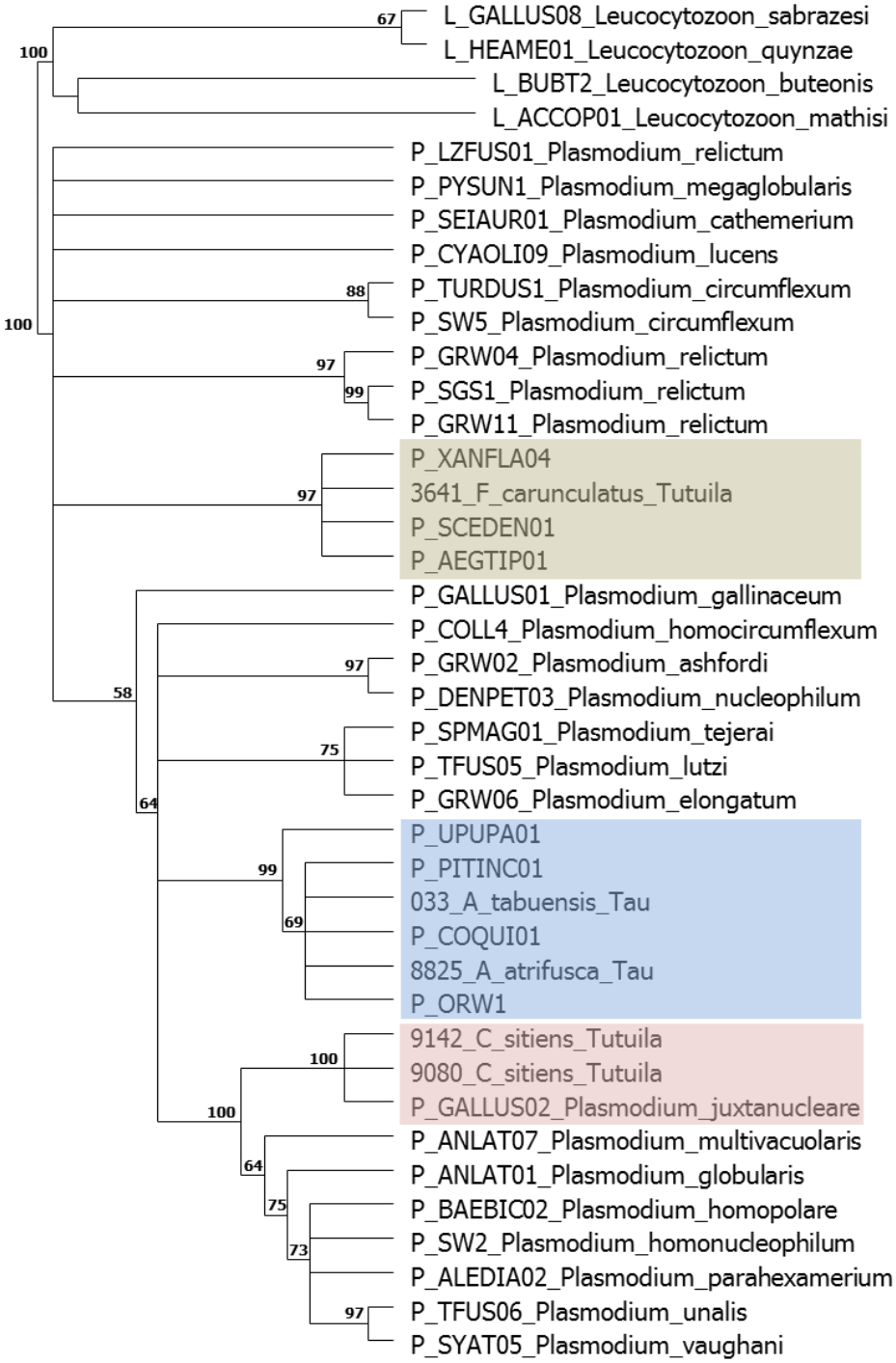


Table 6. Measurements of *Trypanosoma* sp. from American Samoa, *T. corvi* from a Rufous Treepie, *Dendrocitta vagabunda* (Nandi and Bennett 1994), *T. avium* from European Pied Flycatcher, *Ficedula hypoleuca* (Bennett et al. 1994), and *T. culicavium* from experimentally infected canaries, *Serinus canaria* (Votýpka et al. 2012). AK = area of kinetoplast; AN = area of nucleus; AT = area of trypomastigote; BW = width of body through center of nucleus; FF = free flagellum; KN = kinetoplast to center of nucleus; NA = center of nucleus to anterior end; PA = total length without free flagellum; PK = posterior end to kinetoplast; PN = posterior end to center of nucleus; BW/PA = body width index; PK/PA, PN/NA, PN/PA 5 nuclear index; PN/KN = kinetoplast index.

| Feature | *T. sp.* American Samoa (n = 10) | | *T. corvi** | *T. avium*[#] | T. *culicavium*^ |
|---|---|---|---|---|---|
| | Range (µm) | Mean ± SD | Range | Mean ± SD | Range (µm) |
| AK | 0.26–0.52 | 0.37 ± 0.09 | nr | 1.1 ± 0.4 | nr |
| AN | 13.84–25.16 | 19.96 ± 3.81 | nr | 15.0 ± 3.6 | nr |
| AT | 101.82–185.73 | 157.54 ± 25.23 | nr | 126.1 ± 24.4 | nr |
| BW | 5.21–8.14 | 6.46 ± 1.01 | 7.0–8.0 | 4.6 ± 1.0 | 3.7–4.1 |
| FF | 2.8–5.4 | 4.18 ± 0.98 | 9.0–11.5 | 7.1 ± 1.2 | 6.8 - 8.0 |
| KN | 8.04–11.28 | 9.38 ± 0.99 | 7.5–9.0 | 11.8 ± 1.9 | nr |
| NA | 23.98–29.77 | 26.83 ± 1.56 | 22.0–23.5 | 26.1 ± 4.1 | nr |
| PA | 43.03–58.09 | 49.87 ± 5.31 | 35.5–37.5 | 51.8 ± 5.2 | 40.7–40.9 |
| PK | 10.94–23.68 | 16.33 ± 3.75 | 3.5–6.0 | 14.1 ± 2.2 | nr |
| PN | 18.63–29.49 | 23.66 ± 3.38 | 12.5–14.5 | 25.7 ± 3.0 | nr |
| AN/AT | 0.09–0.17 | 0.13 ± 0.03 | nr | 0.12 | nr |
| BW/PA | 0.10–0.18 | 0.13 ± 0.02 | nr | 0.09 | nr |
| PK/PA | 0.24–0.41 | 0.33 ± 0.06 | 0.140 | 0.27 | nr |
| PN/KN | 2.04–3.64 | 2.56 ± 0.53 | 1.640 | 0.22 | nr |
| PN/NA | 0.71–1.04 | 0.88 ± 0.12 | 0.580 | 1 | nr |
| PN/PA | 0.41–0.57 | 0.48 ± 0.05 | 0.380 | 0.5 | nr |

*Nandi and Bennett 1994

[#]Bennett et al. 1994

^Votýpka et al. 2012

nr = not reported

Figure 10. *Trypanosoma* from Samoan starlings captured at Faleasao on Ta'u Island (a, b, c, d) and Amalau on Tutuila (e, f). Bar = 10 µm. Note kinetoplast (K), nucleus (N), free flagellum (FF), and undulating membrane (UM). By convention, the free flagellum defines the anterior end of trypomastigotes and continues as the undulating membrane to the kinetoplast. Note pellicular striations (S) that extend from anterior to posterior ends of trypomastigotes.

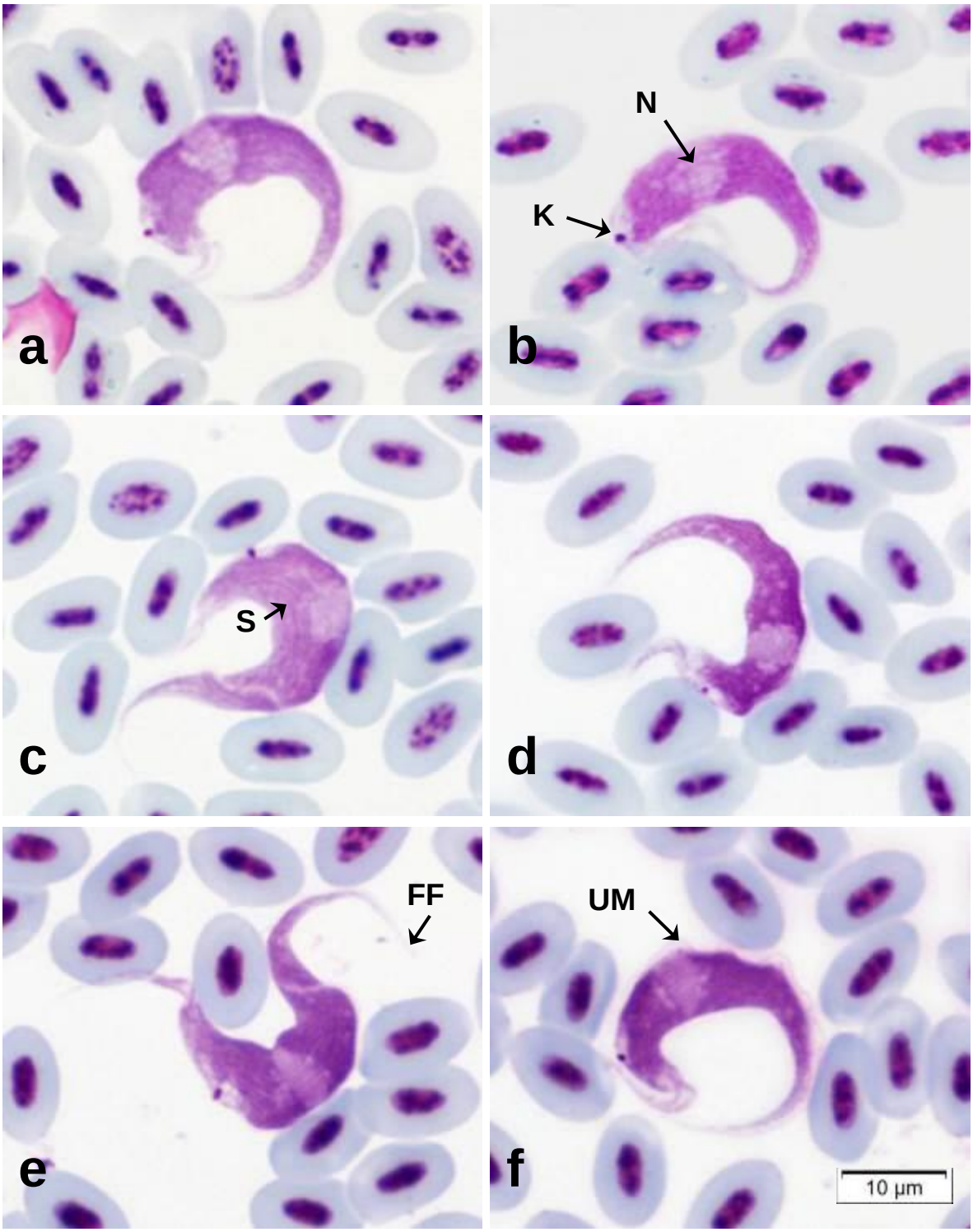


Figure 11. Bayesian inference phylogenetic reconstruction based on 1239 nucleotides of the 16S ribosomal RNA gene of avian *Trypanosoma*. The tree includes sequence data from known morphological species of avian *Trypanosoma* (*T. bennetti*, *T. avium*, *T. culicavium*), sequences from European *Trypanosoma* isolates (Zídková et al. 2012) plus sequences from Samoan starlings (*A. atrifusca*) and Wattled honeyeaters (*F. carunculata*) from Ta'u and Tutuila. The sequences group into three well supported clades (A, B, C). American Samoan sequences fall within Clade C.

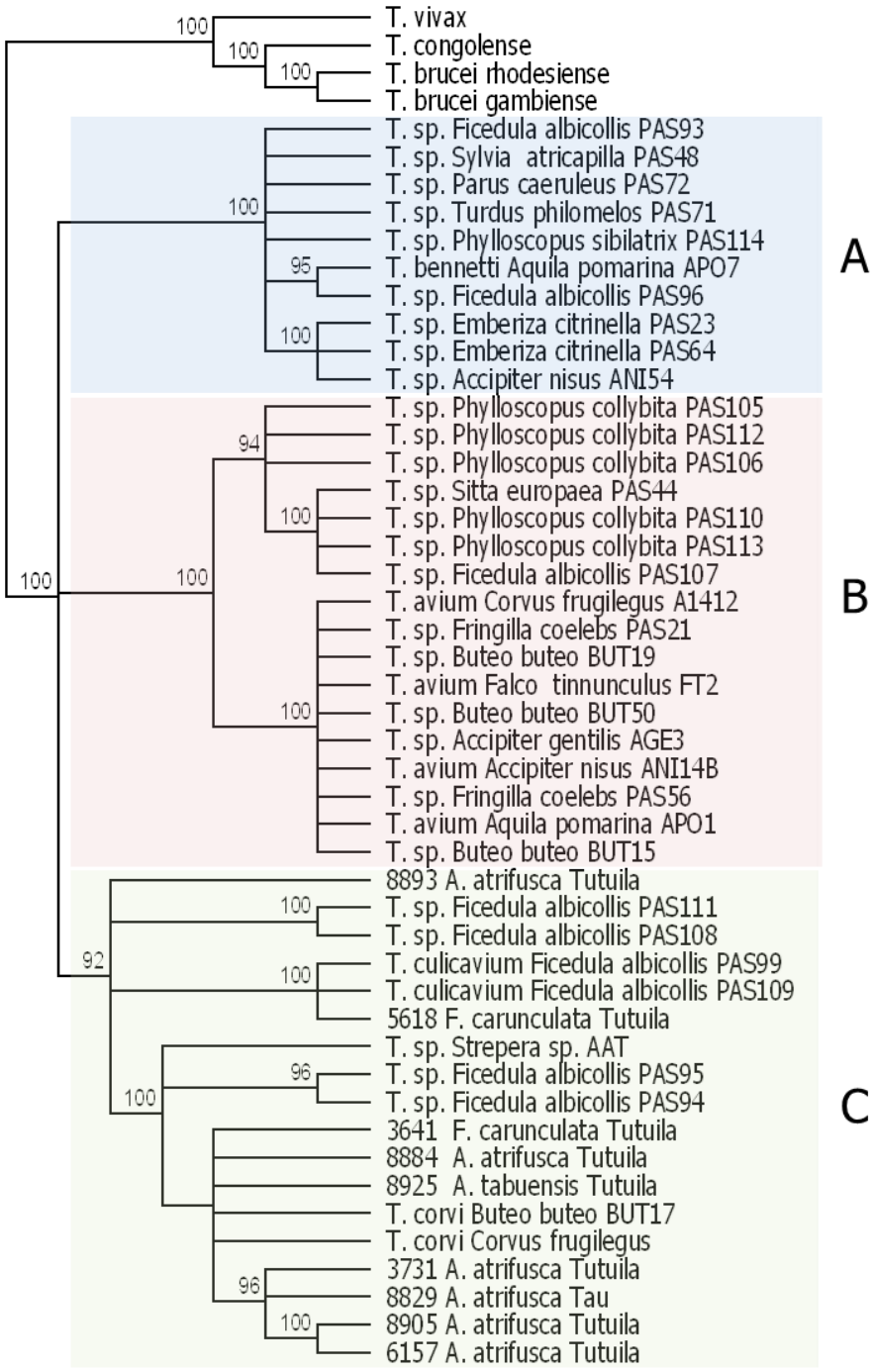


Figure 12. Representative examples of microfilaria from a Polynesian starling. Note sheath (S) that frequently extends from both the blunt anterior (A) and pointed posterior (P) ends of the nematodes.

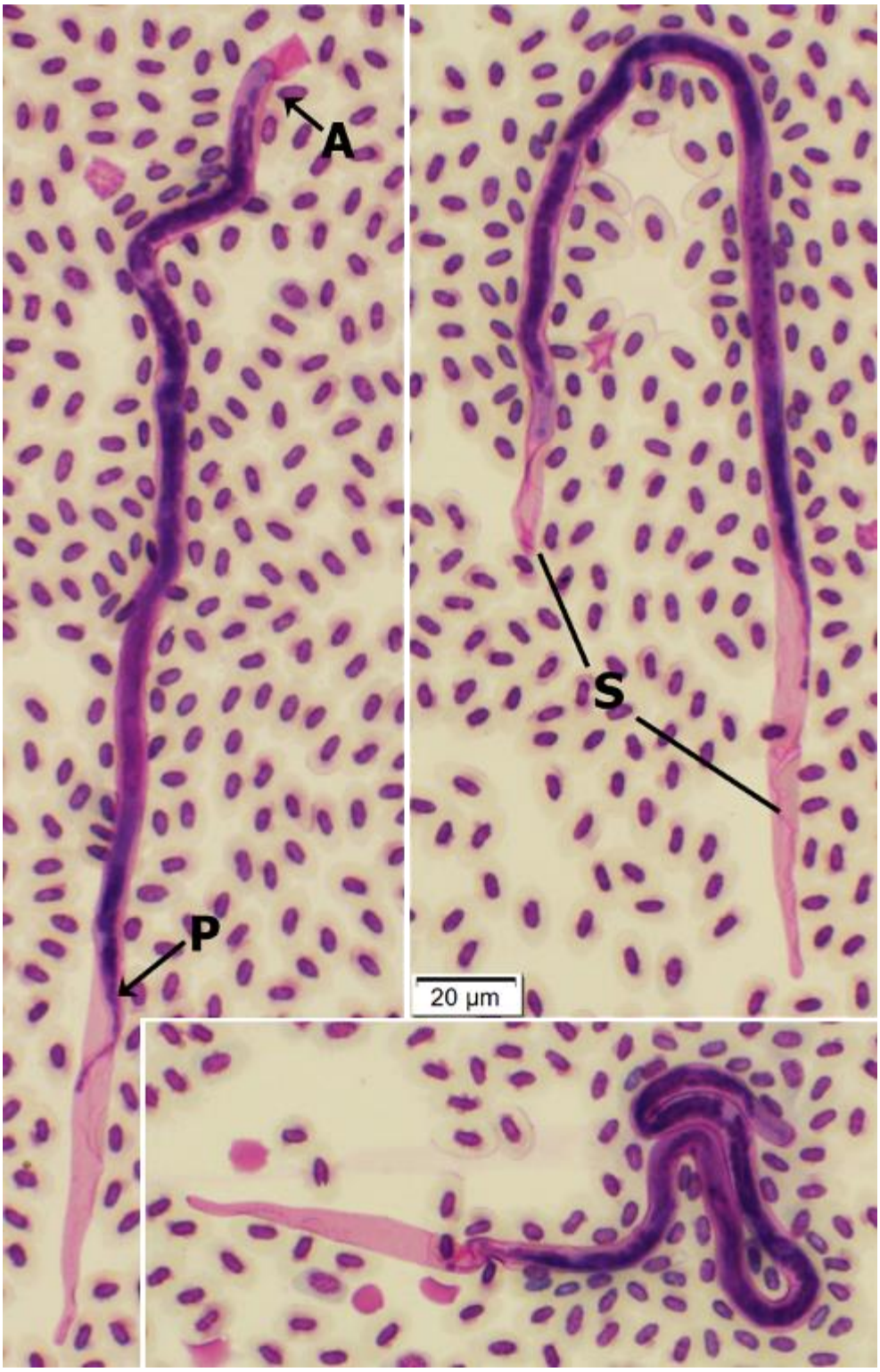
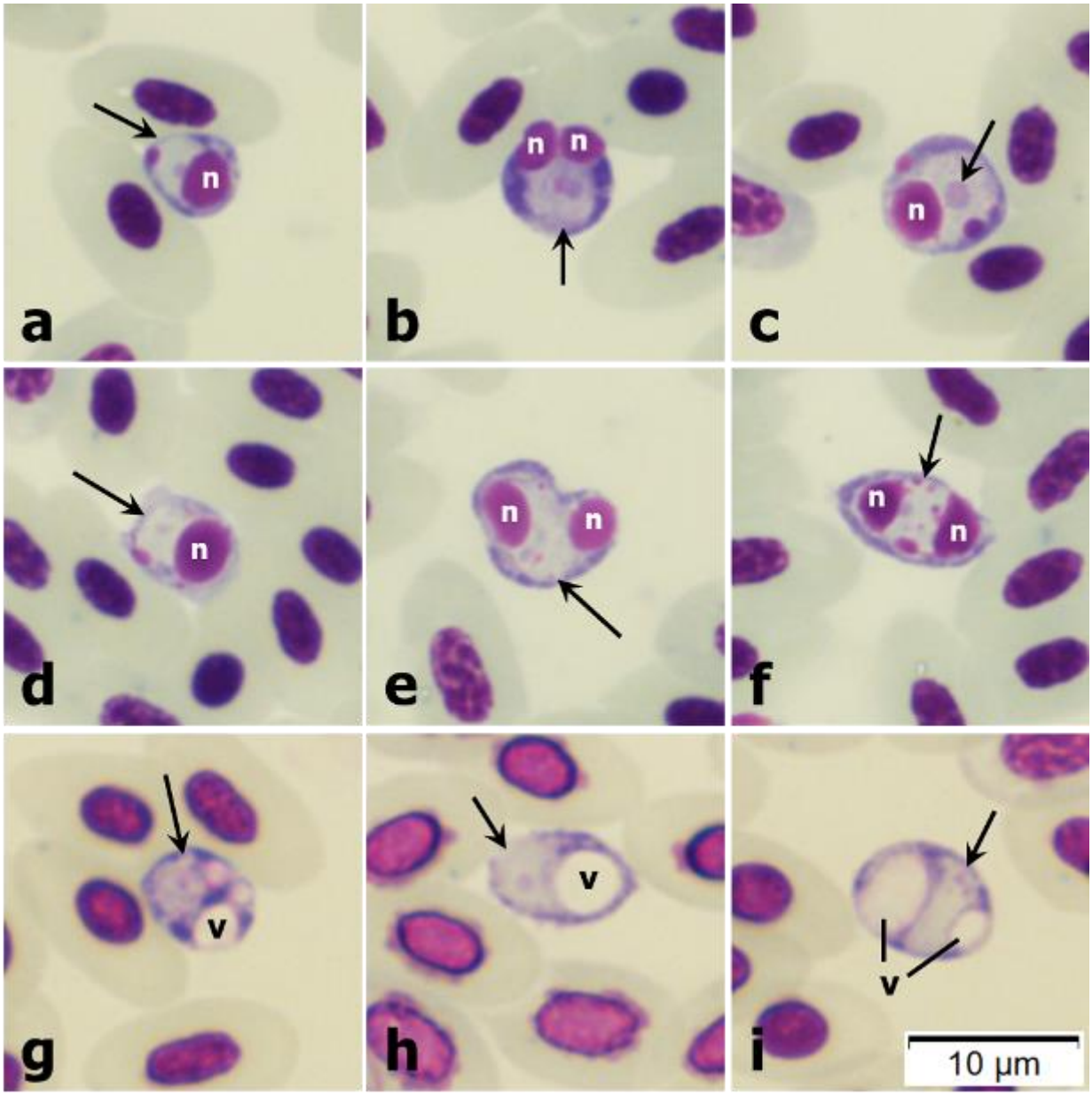


Figure 13. Representative examples of haemococcidians (*Isospora* sp.) from a Polynesian starling. Trophozoites (arrows) are spherical, often distorted in shape by the adjacent host lymphocyte nucleus (n), and may or may not have a visible nucleus (c, arrow). The lymphocyte nucleus eventually degenerates into one or more vacuolated areas (v, g–h) that are adjacent to developing parasites (arrows).



Positive detections were from pooled abdomens and/or thoraxes from either *C. sitiens* (SCENDEN01 and GALLUS02), *C. quinquefasciatus* (SCEDEN01), and pools of mixed *C. sitiens* and *C. annulirostris* (GALLUS02) from Amalau and Olo Ridge on Tutuila Island. We also amplified lineages similar to GALLUS02 from pools of *C. sitiens* collected at Faleasao on Ta`u Island.

Both *C. quinquefasciatus* and *C. sitiens* appear to be able to support complete sporogonic development of SCEDEN01 and lineages similar to GALLUS02 based on positive detections in pooled and individual thoraxes and direct observation of salivary gland sporozoites. Prevalence in mosquito pools ranged from 0.8 to 33% depending on collection location (Table 7). We also observed flagellated protozoa that resembled epimastigotes of *Trypanosoma* sp. in a single *C. sitiens* from Amalau and three *A. oceanicus* from Faleasao. All infected mosquitoes were captured in carbon dioxide-baited CDC traps (Table 7).

**Recaptures**

We recaptured 52 individual birds over the course of the 10-year study, including 29 Wattled honeyeaters, 13 Samoan starlings, six Collared kingfishers, three Polynesian starlings, and one Purple-capped fruit dove. Five birds (three Samoan starlings and two Wattled honeyeaters) acquired infections with *Plasmodium*, one Wattled honeyeater tested negative for *Plasmodium* six months after testing positive for infection with SCEDEN01, and five birds (one Samoan starling and four Wattled honeyeaters) had no change in infection status with *Plasmodium* from eight months to five years after initial capture. One of the Samoan starlings that acquired infection with *Plasmodium* was captured at Amalau in July, 2002 and recaptured almost nine years later in March, 2011. We found evidence of movement between Olo Ridge and Amalau on Tutuila. Three Wattled honeyeaters and four Collared kingfishers were banded at one site and recaptured at the other.

**Lesions**

We occasionally observed smooth nodular swellings at or near the joint between the distal tarsometatarsus and digits of the feet. Lesions were most common on Wattled honeyeaters (Figure 14) and Samoan starlings, but were observed in fewer than 1% of captures. The swellings were smooth, nodular, and measured from 4–8 mm in diameter, but were unlike the scabby, inflamed and swollen lesions that are typical of cutaneous infections with *Avipoxvirus*.

Table 7. Pooled mosquito collections (*Culex* and *Aedes*) from Amalau, Olo Ridge, and Faleasao that were tested for *Plasmodium*. Separate pools of abdomens and thoraxes were prepared from up to 10 individual mosquitoes and tested separately. Minimum prevalence is based on the assumption that at least one infected mosquito is present in each positive pool. Lineage is based on sequence of a 478 bp region of the parasite mitochondrial cytochrome b gene. *Aedes* (*Finlaya*) may include *A. oceanicus*, *A. samoanus*, and/or *A. tutuilae. Aedes* (*Stegomyia*) may include *A. polynesiensis*, *A. upolensis*, and/or *A. aegypti*. T= one or more positive thoraxes; A = one or more positive abdomens; S= salivary gland sporozoites observed at dissection.

| | Species | Pools | Positive Pools | Total | Minimum Prevalence (%) | Lineage |
|---|---|---|---|---|---|---|
| Amalau | *A. upolensis* | 2 | 0 | 2 | 0 (0/2) | |
| | *A. tutuilae* | 1 | 0 | 2 | 0 (0/2) | |
| | *A. samoanus* | 5 | 0 | 38 | 0 (0/38) | |
| | *A. polynesiensis* | 6 | 0 | 47 | 0 (0/47) | |
| | *A. oceanicus* | 3 | 0 | 18 | 0 (0/18) | |
| | *A.* (*Stegomyia*) | 3 | 0 | 21 | 0 (0/21) | |
| | *A.* (*Finlaya*) | 5 | 0 | 39 | 0 (0/39) | |
| | *C. annulirostris* | 3 | 0 | 5 | 0 (0/5) | |
| | *C. quinquefasciatus* | 3 | 1 | 3 | 33 (1/3) | SCEDEN01$^{T}$ |
| | *C. sitiens/annulirostris* | 4 | 1 | 4 | 25 (1/4) | GALLUS02$^{S}$ |
| | *C. sitiens* | 3 | 1 | 17 | 5.9 (1/17) | GALLUS02$^{T}$ |
| Olo Ridge | *A. samoanus* | 1 | 0 | 4 | 0 (0/0) | |
| | *A. polynesiensis* | 4 | 0 | 26 | 0 (0/26) | |
| | *A. oceanicus* | 1 | 0 | 1 | 0 (0/1) | |
| | *A.* (*Stegomyia*) | 1 | 0 | 3 | 0 (0/3) | |
| | *A.* (*Finlaya*) | 3 | 0 | 15 | 0 (0/15) | |
| | *C. quinquefasciatus* | 8 | 2 | 8 | 25 (2/8) | SCEDEN01$^{T}$ |
| | *C. sitiens/annulirostris* | 6 | 0 | 6 | 0 (0/6) | |
| | *C. sitiens* | 33 | 5 | 264 | 1.9 (5/264) | GALLUS02$^{T, A}$ |
| | | 33 | 2 | 264 | 0.8 (2/264) | SCEDEN01$^{T}$ |
| Faleasao | *A. upolensis* | 1 | 0 | 9 | 0 (0/9) | |
| | *A. tutuilae* | 2 | 0 | 4 | 0 (0/4) | |
| | *A. polynesiensis* | 2 | 0 | 19 | 0 (0/19) | |
| | *A. oceanicus* | 70 | 0 | 688 | 0 (0/688) | |
| | *A.* (*Stegomyia*) | 3 | 0 | 27 | 0 (0/27) | |
| | *A.* (*Finlaya*) | 30 | 0 | 300 | 0 (0/300) | |
| | *C. annulirostris* | 7 | 0 | 52 | 0 (0/52) | |
| | *C. quinquefasciatus* | 5 | 0 | 5 | 0 (0/5) | |
| | *C. sitiens* | 24 | 0 | 43 | 4.7 (2/43) | GALLUS02$^{T,A}$ |

Figure 14. Nodules (arrows) on feet of two Wattled honeyeaters. The nodules are not scabby or characteristic of *Avipoxvirus* infections, and near the distal end of the tarsometatarsus or at the joint between the tarsometatarsus and digits of the feet.

## DISCUSSION

### Diversity

Little is known about the distribution of avian malaria (*Plasmodium* spp.) and other vector-borne avian blood parasites in the south-central Pacific. The natural occurrence of mosquitoes and other biting flies on island archipelagos throughout this region makes it possible that parasites occur here as part of their indigenous and endemic forest bird communities (Evenhuis 1989), but the absence of detailed survey data from points east of Vanuatu, the Solomon Islands and New Zealand make it difficult to define whether there are eastern limits to the distribution of vector borne avian diseases or geographic differences in their diversity (Laird 1950, 1953, 1960). While there have been a number of recent studies documenting diversity of haemosporidian parasites from New Guinea (Beadell et al. 2004), New Caledonia (Ishtiaq et al. 2010), and Vanuatu (Ishtiaq et al. 2010, Olsson-Pons et al. 2015), plus isolated reports of *P. relictum* from Fiji, the Cook Islands, French Polynesia and the Marquesas Islands (Ishtiaq et al. 2006, Beadell et al. 2006), the central South Pacific has remained largely unexplored in spite of the high risk that many of these archipelagoes may face from introduced diseases and disease vectors. Jarvi et al. (2003) documented presence of a *Plasmodium* species on Tutuila Island in American Samoa by PCR and reported prevalence in the forest bird community at Amalau and three other locations, but was unable to identify the parasite based on sequence data from ribosomal and nuclear genes. Atkinson et al. (2006) conducted a wider survey in American Samoa based on microscopy of Giemsa-stained blood smears and reported a diverse parasite fauna based on widespread prevalence of *Plasmodium*, trypanosomes, filarial worms, and haemococcidians and absence of detectable pathogenicity. Low intensity of infection made it difficult to make definitive identifications, but at least one species of *Plasmodium* with morphological affinities to *P. circumflexum*, a *Trypanosoma* species with morphological affinities to *T. avium*, and undetermined species of filarial worms (microfilariae) and haemococcidians were widespread on Tutuila, Ofu, Olosega, and Ta'u Islands. While these studies provided the first detailed look at hematozoan communities from the central Pacific, their methodology was unable to provide more definitive identifications or reveal cryptic diversity that might be missed by microscopy.

*Plasmodium:* We used a combination of both microscopy and molecular methods to identify three taxonomically unique lineages of avian *Plasmodium* in American Samoa based on cytochrome b sequence. Two of the three lineages of *Plasmodium*, SCEDEN01 and ORW1, have broad host ranges and broad distributions throughout Australasia and Europe, although ORW1 is more widely distributed (11 different hosts, 10 genera, 9 families, 4 orders) than SCEDEN01 (6 hosts, 6 genera, 5 families, 1 order) (MalAvi, http://mbio-serv2.mbioekol.lu.se/Malavi/). While ORW1 has been reported from the United Kingdom, Kyrgyzstan and Russia (Siberia), it is primarily an Australasian parasite, with detections in India, Myanmar, Australia, Japan, and Papua New Guinea. SCEDEN01 has a more limited geographic range, with previous reports only from Australia. Neither lineage has been associated with a described species of *Plasmodium* and observations from this study are the first to document their morphology and placement within the subgenus Giavannolaia. This subgenus includes both *P. circumflexum* and *P. homocircumflexum* (Valkiūnas 2005, Palinauskas et al. 2015), which are the closest in morphology to both lineages (Atkinson et al. 2006, this study), but more distantly related based on cytochrome b sequence (Figure 9). Furthermore, lineage ORW1 appears to differ morphologically from both *P. circumflexum* and SCEDEN01 by having filamentous and pointed cytoplasmic processes on trophozoites, immature meronts and gametocytes.

We detected two additional lineages in salivary gland dissections and PCR amplifications from pooled and individual *C. sitiens* and *C. annulirostris* that are closely related to GALLUS02. While relatively widespread and present in vector populations on both islands, we did not detect these lineages in the forest bird community. The cytochrome b sequence for GALLUS02 has been identified as a barcode for *P. juxtanucleare*, a parasite that is common in chickens and other galliform hosts in southeast Asia (Bennett et al. 1966) and the Neotropics (Valkiūnas 2005). *Culex sitiens* is a competent vector of this species in Malaysia (Bennett et al. 1966). We suspect that domestic and feral chickens that are common throughout American Samoa may be harboring this parasite. New World isolates of *P. juxtanucleare* can be pathogenic in domestic fowl, but Asian strains of the parasite are generally less virulent (Valkiūnas 2005). Systematic sampling of domestic poultry will help to verify presence of this parasite and determine its range in American Samoa.

SCEDEN01 comprised approximately 80% of infections on both Tutuila and Ta'u islands, with highest prevalence of infection in Wattled honeyeaters. By contrast, ORW1 was primarily a parasite of Samoan and Polynesian starlings, with only a single detection in a Wattled honeyeater. It is unclear whether this difference in host range is a result of physiological differences in host specificity or behavioral or ecological factors associated with exposure to vectors. While we occasionally found double peaks during editing of sequencing chromatograms that might indicate double infections with both parasites, there was no consistent pattern in their occurrence that would suggest evidence of coinfection.

We detected SCEDEN01, the most common lineage of *Plasmodium* in Samoan forest birds, in individual and pooled thoraxes of *C. sitiens* and *C. quinquefasciatus* collected at Amalau and Olo Ridge. Detection of parasites in thoraxes of these mosquitoes suggests that the parasite can complete sporogony and invade salivary glands that are located in the anterior thorax. Definitive proof of their role as vectors of this lineage requires both salivary gland dissections to demonstrate sporozoites within the glands and transmission experiments that involve the bite of infected mosquitoes. We did not detect any mosquito infections with ORW1, but this may be because of the much lower prevalence of this lineage relative to SCEDEN01.

With the exception of a single SNP in an isolate of ORW1 from a Samoan starling from Ta'u, sequence data from more than 100 isolates of both ORW1 and SCEDEN01 exhibited no variation between or within islands in American Samoa. The minor amount of variability we detected in ORW1 might suggest that it has been present on the archipelago longer than SCEDEN01 or that multiple introductions of the lineage have occurred through movement of migratory birds. This particular SNP is more common in independent Samoa (C. Atkinson, unpublished data), which suggests that interisland movement within the Samoan Archipelago is possible. We detected a single Long-tailed cuckoo that was positive for *Plasmodium* with primers that amplify parasite ribosomal genes, but were unable to amplify parasite mitochondrial cytochrome b sequence and determine its relationship to either SCEDEN01 or ORW1. This migrant breeds in New Zealand and winters throughout islands of the SW Pacific and may play a significant role in moving parasites throughout Oceania. Other potential migrants include Lesser golden plovers, Wandering tattlers, Ruddy turnstones and other shorebirds, but prevalence of malaria in these species is generally low (Mendes et al. 2005).

The most similar lineages in MalAvi to SCENDEN01 (XANFLA04, AEGTIP01) and ORW1 (UPUPA01, PITINC01, COQUI01) differ by < 2% and may belong to the same species of *Plasmodium*, but this will require detailed studies of their morphology and life history

characteristics (Valkiūnas 2005). With the exception of COQUI01, they have similar Australasian distributions with reports in Australia and Myanmar. By contrast, COQUI01 has been reported from Sub-Saharan Africa.

Overall prevalence of infection with *Plasmodium* was 15%, almost 25% lower than reported by Jarvi et al. (2003). Patterns of infection were similar between the two studies, however, with highest prevalence in Samoan and Polynesian starlings, Wattled honeyeaters, and Cardinal honeyeaters. These differences probably stem from different diagnostic primers that were used in the two studies since the earliest samples collected by this study (2001) are only five years later than those collected by Jarvi et al. (2003). Jarvi et al. (2003) used a nested PCR based on ribosomal primers developed by Feldman et al. (1995) that has not been widely adopted by the research community. Additional work to compare sensitivity and specificity of these two approaches would help to determine why prevalence differed as much as it did between the two studies.

Jarvi et al. (2003) did not detect serological cross reactivity with Hawaiian isolates of *P. relictum* during analysis of plasma samples from Samoan birds. This observation supports our finding of lineages of *Plasmodium* that are taxonomically distinct from *P. relictum* and provides additional evidence that *P. relictum* has not been introduced to the archipelago.

*Trypanosoma*: We observed what appears to be a single species of *Trypanosoma* in Samoan and Polynesian starlings, Wattled honeyeaters, and Fiji shrikebills based on both morphology of circulating trypomastigotes and small subunit ribosomal RNA sequence amplified from blood samples. Our Bayesian phylogenetic analysis placed the parasites within the same clade as *T. corvi* and *T. culicavium*, one of three polyphyletic clades of avian trypanosomes identified by Zídková et al. (2012), but we were unable to assign the parasites to a specific species. The taxonomy of avian trypanosomes is still poorly understood because of incomplete species descriptions in the early literature, a proliferation of names based on the early belief that the parasites are host specific, and the absence of molecular markers that can distinguish individual species (Baker 1976, Valkiūnas et al. 2011). Valkiūnas et al. (2011) and others have recommended that most named species should be considered *nomina dubia*, with only provisional status until more detailed morphometric and experimental studies can be completed. Differences in size (PA, Table 6) and location of the kinetoplast and nucleus (PK, PN, Table 6), among the Samoan parasites and *T. corvi*, *T. culicavium*, and *T. avium* suggest that the Samoan trypanosome is taxonomically distinct from these three species, but morphometric comparisons and amplification of molecular markers from other named species are needed to determine whether the Samoan parasites warrant description as a new species.

*Filarial Worms*: A single species of avian filarial worm appears to be present in American Samoa based on morphology of microfilariae, but further description and identification will require recovery of adult filarial worms. We detected microfilariae in a Purple Capped Fruit Dove, a White-rumped swiftlet, and a Collared kingfisher, but prevalence was highest in Samoan and Polynesian starlings and Wattled honeyeaters (Table 5). The microfilariae that we observed were sheathed and consistent in size and morphology with microfilariae that have been described from species of *Pelecitus*, *Struthiofilaria* and *Eulimdana* (Bartlett 2008). Adult filarial worms of these species are found in the body cavity (*Struthiofilaria*), subcutaneous connective tissue of the head and neck or connective tissue around the trachea, esophagus, or crop (*Eulimdana*) or in subcutaneous connective tissue near joints of the legs, feet or toes (*Pelecitus*) (Bartlett 2008). We observed unusual swellings associated with joints of the feet in a small

number of Wattled honeyeaters which may be consistent with presence of adult filarid nematodes (Figure 14), but additional work is needed to verify this hypothesis. With few exceptions, these parasites are generally viewed as non-pathogenic in avian hosts (Bartlett 2008), and we did not observe clinical signs of infection.

*Haemococcidians*: We detected haemococcidians (*Isospora* = *Atoxoplasma*) in a Blue-crowned lorikeet, a White-rumped swiftlet, and in Samoan and Polynesian starlings and Wattled honeyeaters. These parasites complete their life cycle within epithelial cells and mononuclear lymphocytes in the intestinal mucosa, eventually producing thin walled oocysts that are excreted with the feces (Greiner 2008). Oocysts complete asexual sporogony in the external environment, and eventually become infective to new hosts through ingestion of contaminated food, water or soil. During the intestinal phase of the infection, mononuclear lymphocytes containing merozoites may spill into the circulation where they can be detected on blood smears. The presence of these circulating lymphocytes led to confusion in early studies about their taxonomy, the potential role that blood-sucking arthropods may play in their transmission, and classification as a distinct genus (*Atoxoplasma*). It is now recognized that the life cycle is completed through ingestion of fecal oocysts rather than passage through an arthropod vector. Generic classification of these parasites was moved to the genus *Isospora*, with *Atoxoplasma* retained as a junior objective synonym (Schrenzel et al. 2005, Barta et al. 2005).

Prevalence and intensity of infection with *Isospora* was low (< 5%) among the five species of forest birds that were positive, but observations based solely on blood smears probably underestimate the true prevalence of infection. Infected mononuclear cells (monocytes, lymphocytes, heterophils), typically had dark bluish cytoplasm and darkly staining, pycnotic nuclei that appeared to become vacuolated as parasite development progressed. These changes suggest that infected mononuclear host cells were degenerating. While epizootic outbreaks and mortality from infections with these parasites have been detected in wild birds (Khan and Desser 1971), reports are rare (Greiner 2008) and we did not detect clinical signs of infection when birds were handled for banding and blood collection. Further description and identification of the parasites will require examination of fecal oocysts and intestinal tissue for developmental stages of *Isospora*.

**Vectors**

American Samoa has an indigenous and introduced fauna of biting flies, including mosquitoes (*Aedes nocturnus*, *A. oceanicus*, *A. samoanus*, *A. tutuilae*, *A. aegypti*, *A. polynesiensis*, *A. upolensis*, *Coquillettidia samoaensis*, *Culex quinquefasciatus*, *C. sitiens*, *C. annulirostris*; Ramalingam 1976, Evenhuis and Gon 1989*)*, ceratopogonid flies (*Culicoides mollis*, *C. polynesiae*, *C. samoensis*, and *C. insulanus*; Wirth and Arnaud 1969, Debenham 1989), ectoparasitic avian hippoboscid flies (*Ornithoctona plicata*, *Ornithoica pusilla*, *Ornithophila metallica*; Ma 1989), ectoparasitic mammalian nycterbiid flies (*Cyclopodia inclita*; Ma 1989), and a single species of tabanid fly (*Tabanus samoensis*; Burger 1991).

Mosquito transmission of human and domestic animal disease is well documented in American Samoa with both ongoing and emerging reports of lymphatic filariasis (*Wuchereria bancrofti*), Dengue Fever, Ross River Virus, and Chikungunya virus in the human population, and heartworm (*Dirofilaria immitis*) in domestic dogs (Mladonicky et al. 2009, Duncombe et al. 2013, Tesh et al. 1981, CDC 2015, Chambers et al. 2009). We were able to establish that the indigenous mosquito, *C. sitiens*, and introduced *C. quinquefasciatus*, are likely vectors of avian malaria in the territory through both salivary gland and midgut dissections and PCR

amplification of parasite mitochondrial genes in pooled thoraxes. Both species of *Culex* are proven vectors of avian malaria in Asia, Hawaii, and other parts of the world (Bennett and Warren 1966, LaPointe et al. 2005).

One or more species of mosquitoes are possible vectors of avian filarial worms, but these nematodes are transmitted by a wide variety of biting arthropods, including fleas, simuliid (black) flies, and ceratopogonid flies (Bartlett 2008). Additional analysis of pooled mosquito samples with filarial specific PCR primers may help to determine how they are transmitted in American Samoa, but this was outside the scope of our study. Avian trypanosomes are also transmitted by a wide variety of arthropods, including mosquitoes, ectoparasitic hippoboscid flies, black flies, and ceratopogonid flies (Baker 1976). We observed flagellated epimastigotes during a midgut dissection of a single *C. sitiens* collected in Amalau Valley. PCR amplification with primers that amplify flagellate ribosomal DNA produced products of the expected sizes, but sequence data did not support any relationship to avian trypanosomes. The parasites may have been a species of *Crithidia*, a common endosymbiotic flagellate in the digestive tracts of insects.

Reports of at least four species of *Culicoides* and several species of ectoparasitic hippoboscid flies in American Samoa opens the possibility that transmission of one or more species of *Haemoproteus* could be supported. No known ceratopogonid-transmitted diseases have been reported in the archipelago, however, and we did not detect *Haemoproteus* by blood smears or PCR.

**Climate**

The seasonal climate in American Samoa is relatively constant with daily minimum and maximum temperatures that range between 24°C and 32°C throughout the year. Mean high temperatures are several degrees warmer, mean daily wind speed is lower, and mean daily rainfall is higher during the summer season (November–April) than in the winter (May–October). These environmental conditions are capable of supporting year round transmission of avian malaria (LaPointe et al. 2010). The relatively high prevalence of infections and absence of significant seasonal or yearly changes in prevalence are not surprising, especially given the chronic nature of infections with avian hematozoa that may persist for the lifetime of infected birds. Atkinson et al. (2006) observed a significant increase in filarial infections following passage of Hurricane Olaf and speculated that catastrophic climatic events could lead to increases in transmission and prevalence of infection. We did not see this trend in data that were collected over a longer period of time, but sampling may not have been frequent enough to detect changes that may have been significant over short periods of time. More detailed studies that measure incidence of infection and changes in vector populations may help to determine whether environmental factors vary enough in American Samoa to have significant effects on parasite transmission.

**Land Use**

We observed significant island and island*land use interactions on prevalence of avian hematozoans. These effects were driven primarily by the higher overall prevalence of infection on Ta'u Island relative to Tutuila and the significantly higher prevalence of infection on agricultural land on Ta'u relative to remote forest habitat. Similar trends were evident on Tutuila between agricultural sites at Amalau and forest habitats on Olo Ridge. These differences were not significant, however, because the sites were close enough to each other to allow movement of birds between the two locations. Our findings are in agreement with those of Mendenhall et al. (2013) who observed a significant increase in prevalence of avian malaria in fragmented

agricultural habitats with increased forest edge and decreased forest area. This increase is presumably associated with increases in vector populations that may follow creation of larval habitat in agricultural sites and increased movement of vectors in more open, fragmented agricultural habitats. If conversion of forest habitats to agricultural use is extensive, it may lead to increased spread of vector-borne parasites to remote areas through movement of species, e.g. Polynesian starlings, that favor agricultural areas but move freely between both habitats.

While we have no indication that higher prevalence of infection is limiting forest bird populations in agricultural habitats on Tutuila or Ta'u, both the physical proximity of agricultural areas to roads and human development and higher transmission rates in these locations may help to speed establishment and spread of introduced vector borne pathogens.

**Conservation Significance**

Our findings provide the first detailed records of avian hematozoan parasites from the Central Pacific, provide baseline data on the number and diversity of vector-borne parasites in the archipelago, and build on earlier observations by Jarvi et al. (2003) and Atkinson et al. (2006). We observed a relatively stable host-parasite community over the duration of the 10-year study and suggest that the parasite fauna in Samoan forest birds is indigenous given the high prevalence of chronic infections, absence of recent range contractions and declines, and presence of an indigenous vector fauna.

We amplified *Plasmodium* sequence from both *C. sitiens* and *C. quinquefasciatus* that matches mitochondrial sequence of *P. juxtanucleare*, but additional work is needed to verify presence of the parasite in domestic poultry on the island. Since chickens are not indigenous to the islands, this parasite is most likely an introduction to American Samoa that reached the islands through movement of domestic poultry. We found no evidence that the parasite has moved from potential chicken (galliform) reservoirs into more distantly related passerine hosts, possibly because of the high host specificity of this species of *Plasmodium* (Valkiūnas 2005).

One of the most significant findings of the study was the apparent absence of both *P. relictum* and *Avipoxvirus*, two introduced pathogens that have contributed to the extinction of native Hawaiian forest birds. *Plasmodium relictum* has been reported in introduced Mynas (*Acridotheres tristis*) in the Cook Islands (Ishtiaq et al. 2006), introduced Red-browed firetails (*Neochmia temporalis*), Silvereyes (*Zosterops lateralis*) and Common mynas (*A. tristis*) from Moorea in French Polynesia (Beadell et al. 2006), and in Marquesan reed warblers (*Acrocephalus mendanae*) in the Marquesas Islands (Beadell et al. 2006). Both Common mynas and Jungle mynas (*A. fuscus*) are relatively recent introductions to Tutuila Island and are not yet established on Ta'u Island (Potter 1981, McAllan and Hobcroft 2005). It is possible that *P. relictum* may be present in the Myna population on Tutuila, but the wide host range of this parasite (Beadell et al. 2006) and absence of detection in indigenous forest birds throughout the 10-year period of our study is encouraging and suggests that *P. relictum* has not yet reached the Territory.

In contrast to *Plasmodium*, the closest reports of passerine species of *Avipoxvirus* in the Central Pacific are from New Zealand (Ha et al. 2011), Hawaii (Jarvi et al. 2008), and the Galapagos Islands (Parker et al. 2011), where the virus has had documented effects on native forest birds. We did not observe pox-like lesions on any of the forest birds that we handled and have no evidence that would suggest that it has been introduced to American Samoa. By contrast, fowlpox has been reported in domestic poultry throughout the region, including independent

Samoa, Cook Islands, Kiribati, Fiji, and Tonga, although we can find no reports from American Samoa (Aiolupo 2012, Saville 1994, 1996a, 1996b). While there is some evidence that fowlpox has jumped from domestic poultry to some species of native forest birds in New Zealand (Ha et al. 2011), forest birds (passerine species) in the Hawaiian Islands, the Galapagos Islands and the Canary Islands are infected with distinct passerine forms of *Avipoxvirus* that are unrelated to fowlpox (Jarvi et al. 2008, Parker et al. 2011, Atkinson et al. 2012).

Unlike the Hawaiian Islands, our findings suggest that introduced vector-borne avian diseases may not be a significant problem in American Samoa. The absence of a commercial pet trade in the territory and the continued enforcement of quarantines and restrictions on importation of birds has been an effective barrier against introduction of vector-borne avian diseases that might have significant effects on native and indigenous passerines. Both continued monitoring for new avian disease introductions and additional work on the effects of changing climate and land use practices on transmission of existing vector-borne parasites may help determine whether these factors can lead to host and range shifts and increased pathogenicity in existing parasite communities.

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## APPENDIX I

Appendix 1. Lineages and corresponding GenBank Accession Numbers that were used to generate phylogenetic trees for *Plasmodium* (Figure 9) and *Trypanosoma* (Figure 11). Outgroups in the respective trees include species of *Leucocytozoon* for Figure 9 and mammalian species of *Trypanosoma* for Figure 11.

| Figure | Isolate | GenBank Accession # |
|---|---|---|
| 9 | L_ACCOP01 (*Leucocytozoon mathisi*) | DQ177241 |
| | L_BUBT2 (*Leucocytozoon buteonis*) | EF607293 |
| | L_GALLUS08 (*Leucocytozoon sabrazesi*) | AB299369 |
| | L_HEAME01 (*Leucocytozoon quynzae*) | KF479480 |
| | P_ALEDIA02 (*Plasmodium parahexamerium*) | EU810634 |
| | P_ANLAT01 (*Plasmodium globularis*) | EU770151 |
| | P_ANLAT07 (*Plasmodium multivacuolaris*) | FJ404720 |
| | P_BAEBIC02 (*Plasmodium homopolare*) | AF465555 |
| | P_COLL4 (*Plasmodium homocircumflexum*) | DQ368374 |
| | P_CYAOLI09 (*Plasmodium lucens*) | FJ404707 |
| | P_DENPET03 (*Plasmodium nucleophilum*) | AY640137 |
| | P_GALLUS01 (*Plasmodium gallinaceum*) | AY099029 |
| | P_GALLUS02 (*Plasmodium juxtanucleare*) | AB250415 |
| | P_GRW02 (*Plasmodium ashfordi*) | AF254962 |
| | P_GRW04 (*Plasmodium relictum*) | AF25497 |
| | P_GRW06 (*Plasmodium elongatum*) | DQ368381 |
| | P_GRW11 (*Plasmodium relictum*) | AY831748 |
| | P_LZFUS01 (*Plasmodium relictum*) | JX418224 |
| | P_PYSUN1 (*Plasmodium megaglobularis*) | EU770152 |
| | P_SEIAUR01 (*Plasmodium cathemerium*) | AY377128 |
| | P_SGS1 (*Plasmodium relictum*) | AF495571 |
| | P_SPMAG01 (*Plasmodium tejerai*) | JX272844 |
| | P_SW2 (*Plasmodium homonucleophilum*) | AF495572 |
| | P_SW5 (*Plasmodium circumflexum*) | AF495574 |
| | P_SYAT05 (*Plasmodium vaughani*) | DQ847271 |
| | P_TFUS05 (*Plasmodium lutzi*) | KC138226 |
| | P_TFUS06 (*Plasmodium unalis*) | KC771248 |
| | P_TURDUS1 (*Plasmodium circumflexum*) | AF495576 |
| | P_AEGTIP01 | DQ659581 |
| | P_COQUI01 | GQ150187 |
| | P_ORW1 | AF254963 |
| | P_PITINC01 | AY714204 |
| | P_SCEDEN01 | AY714205 |
| | P_UPUPA01 | EF380118 |
| | P_XANFLA04 | JQ905574 |

| | | |
|---|---|---|
| 11 | *T. avium* (*Corvus frugilegus*) A1412 | U39578 |
| | *T.* sp. (*Accipiter gentilis*)AGE3 | JN006829 |
| | *T.* sp. (*Accipiter nisus*) ANI14A | AY099318 |
| | *T. avium* (*Accipiter nisus*) ANI14B | FJ649483 |
| | *T.* sp. (*Accipiter nisus*) ANI54 | JN006849 |
| | *T. avium* (*Aquila pomarina*) APO1 | AF416559 |
| | *T. bennetti* (*Aquila pomarina*) APO7 | JF778738 |
| | *T.* sp. (*Buteo buteo*) BUT15 | AY099320 |
| | *T. corvi* (*Buteo buteo*) BUT17 | JN006854 |
| | *T.* sp. (*Buteo buteo*) BUT19 | JN006828 |
| | *T.* sp. (*Buteo buteo*) BUT50 | JN006825 |
| | *T. avium* (*Falco tinnunculus*) FT2 | AY099319 |
| | *T. corvi* (*Corvus frugilegus*) | AY461665 |
| | *T.* sp. (*Strepera* sp.) AAT | AJ620557 |
| | *T.* sp. (*Fringilla coelebs*) PAS21 | JN006826 |
| | *T.* sp. (*Emberiza citrinella*) PAS23 | JN006850 |
| | *T.* sp. (*Sitta europaea*) PAS44 | JN006837 |
| | *T.* sp. (*Sylvia atricapilla*) PAS48 | JN006845 |
| | *T.* sp. (*Fringilla coelebs*) PAS56 | JN006827 |
| | *T.* sp. (*Emberiza citrinella*) PAS64 | JN006851 |
| | *T.* sp. (*Turdus philomelos*) PAS71 | JN006847 |
| | *T.* sp. (*Parus caeruleus*) PAS72 | JN006846 |
| | *T.* sp. (*Ficedula albicollis*) PAS93 | JN006852 |
| | *T.* sp. (*Ficedula albicollis*) PAS94 | JN006841 |
| | *T.* sp. (*Ficedula albicollis*) PAS95 | JN006842 |
| | *T.* sp. (*Ficedula albicollis*) PAS96 | JN006848 |
| | *T. culicavium* (*Ficedula albicollis*) PAS99 | HQ107969 |
| | *T.* sp. (*Phylloscopus collybita*) PAS105 | JN006831 |
| | *T.* sp. (*Phylloscopus collybita*) PAS106 | JN006833 |
| | *T.* sp. (*Ficedula albicollis*) PAS107 | JN006835 |
| | *T.* sp. (*Ficedula albicollis*) PAS108 | JN006840 |
| | *T. culicavium* (*Ficedula albicollis*) PAS109 | HQ107966 |
| | *T.* sp. (*Phylloscopus collybita*) PAS110 | JN006836 |
| | *T.* sp. (*Ficedula albicollis*) PAS111 | JN006843 |
| | *T.* sp. (*Phylloscopus collybita*) PAS112 | JN006832 |
| | *T.* sp. (*Phylloscopus collybita*) PAS113 | JN006839 |
| | *T.* sp. (*Phylloscopus sibilatrix*) PAS114 | JN006853 |
| | *T. vivax* (mammals) | EU477537 |
| | *T. brucei rhodesiense* (mammals) | AJ009142 |
| | *T. brucei gambiense* (mammals) | AJ009141 |
| | *T. congolense* (mammals) | AJ009144 |