Description and molecular characterization of a new Leucocytozoon *parasite (Haemosporida: Leucocytozoidae),* Leucocytozoon californicus *sp. nov., found in American kestrels (*Falco sparverius sparverius*)*

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ORIGINAL PAPER

Description and molecular characterization of a new *Leucocytozoon* parasite (Haemosporida: Leucocytozoidae), *Leucocytozoon californicus* sp. nov., found in American kestrels (*Falco sparverius sparverius*)

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Abstract Diurnal raptors in the order Accipitriformes are commonly parasitized with *Leucocytozoon* spp., and the prevalence and intensity of parasitemia are often high. However, for raptors in Falconiformes, several studies have reported relatively low prevalences (1 % or less) of *Leucocytozoon* spp. *Leucocytozoon* parasite pathogenicity has been documented in falcons, but little is known about the diversity, prevalence, and phylogenetic relationships among *Leucocytozoon* species in these predatory birds. The research reported here combines molecular and microscopic techniques to identify and describe *Leucocytozoon* parasites in *Falco sparverius sparverius*, the American kestrel, and place those parasites into a phylogenetic context with leucocytozoids previously found in other diurnal raptors (Accipitriformes), owls (Strigiformes), passerines (Passeriformes), and other bird species. Of 35 American kestrels sampled, 13 birds (37.1 %) were found by PCR to harbor the DNA lineage of a novel species, *Leucocytozoon californicus*. No other *Leucocytozoon* parasite lineages were identified in our sample. Phylogenetic analysis revealed that this parasite clusters more closely with leucocytozoids found in owls and passerines than it does with leucocytozoids found in birds of the genera *Buteo* and *Accipiter* of the order Accipitriformes. This is the first described species of *Leucocytozoon* that parasitizes diurnal raptors in which gametocytes develop exclusively in roundish host blood cells. It is also the first *Leucocytozoon* species that is described and named in birds of the Falconiformes, in which, for unclear reasons, leucocytozoids are significantly less prevalent and less diverse than in raptors with a similar behavioral ecology belonging to the Accipitriformes.

Keywords Haemosporidia · *Leucocytozoon californicus* · American kestrel · Falconiformes

Introduction

Species of *Leucocytozoon* (Haemosporida, Leucocytozoidae) are distributed worldwide, with the exception of Antarctica, and infect a wide variety of birds (Valkiūnas 2005; Forrester and Greiner 2008). One of the species (*Leucocytozoon caulleryi*) belonging to subgenus *Akiba* uses biting midges (family Ceratopogonidae) as vectors. Leucocytozoids of subgenus *Leucocytozoon* are transmitted by black flies of the Simuliidae, in which the sexual process occurs and sporogony completes. These parasites complete tissue merogony and produce gametocytes only in birds; other vertebrates are resistant. Details of development in avian hosts remain unknown for many described species (Valkiūnas 2005). In investigated *Leucocytozoon* parasites, development proceeds according to the following primary scheme: Infected black flies inject

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sporozoites into a bird host while taking a blood meal. The exoerythrocytic (or tissue) merogony occurs in the parenchymal cells of the liver (hepatocytes), macrophages, and various other reticuloendothelial cells, including endothelial cells of the capillaries. Several cycles of exoerythrocytic merogony take place. Mature tissue meronts rupture, releasing merozoites that penetrate blood cells and develop into gametocytes. Gametocyte host cells often assume a fusiform shape, but roundish host cells have also been reported in many *Leucocytozoon* species. Among *Leucocytozoon* species, gametocytes develop in erythroblasts, erythrocytes, and mononuclear leukocytes. Gametocytes are ingested by another black fly where sexual reproduction results in the development of an ookinete, and sporogony occurs. Sporozoites migrate to the vector's salivary glands and will infect the next host bitten by the vector (Valkiūnas 2005; Forrester and Greiner 2008).

Leucocytozoon parasites are closely related to species of the genera *Plasmodium* and *Haemoproteus*; however, leucocytozoids are dissimilar to *Plasmodium* and *Haemoproteus* in that the sexual stage and sporogony occur almost exclusively in black fly vectors of the family Simuliidae. As in *Haemoproteus* spp., asexual reproduction (merogony) of *Leucocytozoon* parasites takes place exclusively in fixed tissues of the vertebrate host. *Plasmodium* and *Haemoproteus* parasites are vectored by mosquitoes and biting midges, respectively, and asexual reproduction of the former parasites takes place both in the blood cells and fixed tissues of the vertebrate host (Forrester and Greiner 2008; Valkiūnas 2005). Additionally, species *of Leucocytozoon* digest hemoglobin completely when they infect red blood cells, which does not occur in pigment-forming haemosporidians (*Plasmodium* and *Haemoproteus*). Phylogenetically, *Leucocytozoon* clade lies basal to *Plasmodium* and *Haemoproteus* clades (Perkins and Schall 2002; Borner et al. 2015), implying that ancestral haemosporidian species lacked merogony in the blood. However, recent work using a Bayesian relaxed molecular clock model suggests that *Plasmodium* spp. may be paraphyletic to species of the other haemosporidian genera, rather than derived, and that *Leucocytozoon* clade lies sister to *Haemoproteus* clade (Outlaw and Ricklefs 2011).

Different species of leucocytozoids are specific to birds on the level of order, family, and in some cases, species (Forrester and Greiner 2008). Diurnal raptors in the family Accipitridae are commonly parasitized with *Leucocytozoon* spp., and prevalence and parasitemia are often high. For example, the prevalence of leucocytozoids was over 94 % in the Palearctic Eurasian sparrowhawk (*Accipiter nisus*) and over 73 % in harriers (*Circus* spp.) (Valkiūnas 2005). This is certainly less so for raptors in the Falconidae family, and several studies have found relatively low prevalences (on the order of 1 %) of *Leucocytozoon* spp., especially compared to the prevalence of *Haemoproteus* infections (Korpimaki et al. 1995; Tella et al. 1996; Dawson and Bortolotti 1999; Dawson and Bortolotti 2001). However, *Leucocytozoon* spp. pathogenicity has been documented in falcons: Clinical signs of infection with *Leucocytozoon* spp., including impaired flight, lack of coordination, weight loss, and mortality, have been noted in wild falcons (Raidal and Jaensch 2000; Tarello 2006), as have histopathological features that were correlated with central nervous disease, blindness, and mortality in Peregrine falcons (*Falco peregrinus*) and Nankeen kestrels (*Falco cenchroides*) (Raidal et al. 1999; Raidal and Jaensch 2000). Although greater study is needed to understand the impact of leucocytozoids on raptor populations, the documented effects on individuals, as well as currently unknown sublethal and interactive effects with other diseases, provide cause for concern and point to a need to better understand the prevalence, diversity, and pathogenicity of leucocytozoids in the world's falcon species.

Information about the diversity, prevalence, and systematics of *Leucocytozoon* species is rapidly growing due to advancements in molecular techniques. Recent molecular work using the *cytochrome b* (*cyt b*) gene has shown *Leucocytozoon* parasites, the most understudied of the avian haemosporidian groups, to be highly diverse and suggests that its taxonomic diversity is probably greater than current classifications indicate (Hellgren 2005; Martinson et al. 2006; Sehgal et al. 2006; Ishak, et al. 2008; Krone et al. 2008; Valkiūnas et al. 2010; Chakarov et al. 2015). Sehgal et al. (2006) found 22 lineages of *Leucocytozoon toddi* in five species of diurnal raptors in California. The authors noted distinct lineages infecting species of the genera *Buteo* versus *Accipiter*: *L. toddi* lineages found in Californian *Accipiter* spp. grouped more closely with those found in European *Accipiter* spp. than they did with *L. toddi* lineages in their sympatric *Buteo* counterparts in California. Further, they found a 10.9 % genetic divergence between parasites inhabiting *Accipiter* and *Buteo* birds. These results suggested that the formerly established *L. toddi* (Greiner and Kican 1977) may be a complex of cryptic species, with different species or subspecies affecting birds of different genera. In fact, Valkiūnas et al. (2010) found that the parasites infecting species of *Accipiter* and *Buteo* were readily distinguishable based on morphology and recommended that they be categorized as *L. mathisi* and *L. buteonis*, respectively.

Despite the common behavioral ecology shared between diurnal birds of prey, recent phylogenetic analyses have separated the Accipitridae and Falconidae families into different taxonomic orders, indicating that Falconiformes is more closely related to Passeriformes and Psittaciformes than to Accipitriformes (Hackett et al. 2008; McCormack et al. 2013; Jarvis et al. 2014). This suggests that species of Falconiformes and Accipitriformes are likely to harbor host-specific leucocytozoids whose phylogenetic relationships mirror those of their hosts.

The research reported here combines molecular and microscopic techniques to identify *Leucocytozoon* parasites in a species of Falconiformes, *Falco sparverius sparverius*, the American kestrel. Our objectives were to identify and morphologically examine *Leucocytozoon* spp. affecting American kestrels and place these parasites into a phylogenetic context with well-documented leucocytozoid lineages previously found in birds of the orders Accipitriformes, Strigiformes, Passeriformes, and Galliformes.

Materials and methods

Study sites and sample collection

Blood samples were collected from 35 breeding American kestrels (*F. s. sparverius*, Federal Bird Banding Permit 22407-F, CA Scientific Collecting Permit SC-10371, UC Berkeley Animal Use Protocol R317) from nest box programs at the University of California Blue Oak Ranch Ecological Reserve (Santa Clara County, California), the Hopland Research and Extension Center (Mendocino County, California), and the Mitsui Ranch (Sonoma Mountain Ranch Preservation Foundation, Sonoma County, California) in April–July of 2009–2012 (Supplementary Table 1). Nest boxes used at all sites were 44 cm tall, 25 cm long, and 22 cm wide, with an entrance hole of 8 cm. The majority of boxes were hung between 2 and 3 m off the ground on Blue Oaks (*Quercus douglasii*) facing either East or North, at an average distance of 500 m apart. Oak grassland and open pastures represented the main habitat type for all three sites, with seasonal streams and small ponds present within one to two miles of all nests sampled.

Adult *F. s. sparverius* were trapped by hand in the box during egg incubation. Each bird was banded with a unique US Geological Survey band, and morphological and genetic data were taken. We obtained approximately 150 μL of blood from each bird via brachial venipuncture and prepared two to three blood smears using a drop of blood on each slide. Slides were air dried, fixed in absolute methanol, and stained with Giemsa as described by Valkiūnas (2005). Remaining blood was stored in 1 mL of lysis buffer (10 mM Tris–HCl pH 8.0, 100 mM EDTA, 2 % SDS) at ambient temperature while in the field and then preserved at −20 °C when returned to the laboratory.

Blood film examination

Morphometric data collection and characters used for the species description were derived from blood smears visualized under an Olympus BX51 light microscope equipped with Olympus DP12 digital camera and imaging software. Olympus DP-SOFT was used to examine slides, to prepare illustrations, and to take measurements. Approximately 100–150 fields were examined at low magnification (×400), and then at least 100 fields were studied at high magnification (×1000). Intensity of parasitemia was estimated as a percentage by counting the number of parasites per 1000 red blood cells or per 10000 erythrocytes if infections were light (<0.1 %). To determine the possible presence of co-infections with other haemosporidian parasites in the type material of new species, the entire blood films from the hapantotype and parahapantotype series were examined microscopically at low magnification. The morphometric features studied were those defined by Valkiūnas (2005) and are listed in Table 1. Student's *t* test for independent samples was used to determine statistical significance between mean linear parameters. A *P* value of 0.05 or less was considered significant.

Table 1 Morphometric parameters of mature gametocytes and host cells of *Leucocytozoon californicus* sp. nov. from the blood of the American kestrel *Falco sparverius sparverius*

Feature	Measurements (μm)[a]
Macrogametocytes	$n = 21$
Length	10.3–15.1 (13.3 ± 1.1)
Width	9.9–13.8 (11.5 ± 0.9)
Area	84.9–147.6 (118.9 ± 13.1)
Parasite nucleus	
Length	3.7–6.4 (4.6 ± 0.6)
Width	2.0–3.7 (2.7 ± 0.5)
Area	7.2–13.7 (10.8 ± 1.8)
Host-cell nucleus	
Length	6.5–18.1 (11.9 ± 2.9)
Width	1.5–4.9 (3.4 ± 0.9)
Area	16.1–45.5 (29.4 ± 8.0)
Host-cell parasite complex	
Area	107.6–197.0 (161.4 ± 25.3)
Microgametocyte	$n = 8$
Length	10.5–12.7 (11.9 ± 0,9)
Width	7.6–10.8 (9.6 ± 1.0)
Area	67.4–99.8 (87.9 ± 10.0)
Parasite nucleus	
Length	9.3–11.1 (10.0 ± 0.8)
Width	3.1–9.2 (6.3 ± 1.9)
Area	28.1–63.8 (48.9 ± 12.4)
Host-cell nucleus	
Length	12.9–15.6 (14.0 ± 1.3)
Width	3.4–4.5 (4.0 ± 0.5)
Area	32.4–46.4 (38.2 ± 5.6)
Host-cell parasite complex	
Area	102.0–205.9 (141.7 ± 37.4)

[a] Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation

DNA extraction, PCR amplification, and sequencing

Cellular DNA was extracted from 25 μL of blood using the QIAGEN DNeasy kit (QIAGEN Inc.). Nested PCR reactions were used to amplify a portion of the *cyt b* gene, following the protocol described in Sehgal et al. (2006). PCR products from positive infections were purified using ExoSap (following the manufacturer's instructions, United States Biochemical Corporation, Cleveland, Ohio). Bidirectional sequencing of the PCR fragments was performed using the primers LeucoF (TCT TAC TGG TGT ATT ATT AGC AAC) and LeucoR (TCT TAC TGG TGT ATT ATT AGC AAC) using an ABI Prism 3100 automated sequencer (Applied Biosystems, Inc., Foster City, California). Sequences were edited using Sequencher 4.8 (GeneCodes, Ann Arbor, MI). We then identified sequences to genus by identifying their closest sequence matches in GenBank via the National Center for Biotechnology Information (NCBI) nucleotide Basic Local Alignment Search Tool (BLAST) search (Altschul et al. 1990).

Phylogenetic analysis

We based our phylogenetic analysis of *Leucocytozoon* spp. on the *cyt b* gene sequence (464 bp) found in the individuals in our *F. s. sparverious* sample as well as 21 *Leucocytozoon* sequences found in Accipitriformes, Strigiformes, Passeriformes, and Galliformes birds downloaded from GenBank (listed in Supplementary Table 2). Sequences were aligned and edited using Seaview Galtier et al. (1996). Phylogenetic relationships were analyzed using the best-fit GTR + G model of molecular evolution as calculated with MrModeltest Nylander et al. (2004) and implemented in MrBayes version 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). *Plasmodium ashfordi* was used as outgroup. Two Markov Chain Monte Carlo (MCMC) simulations were run simultaneously for 10 million generations, sampling every 200 generations. The first 25,000 (25 %) trees were discarded from the sample as the burn-in period, and the remaining trees were used to construct a majority rule consensus tree and to calculate the posterior probabilities of the individual clades. The sequence divergence between the different lineages was calculated using the GTR + G model of substitution, implemented in the program PAUP (Swofford 2001).

Results

Of 35 American kestrels sampled, 13 birds (37.1 %) were determined by PCR to harbor identical sequences of a novel blood parasite species, *Leucocytozoon californicus* (see description below). No other *Leucocytozoon* lineages were detected. Four of the Giemsa-stained smears from the 13 birds that were positive by PCR were of sufficient quality to allow confirmation by microscopy. Microscopic examination also revealed the presence of one morphotype in the PCR-positive birds. The *cyt b* sequence from *Leucocytozoon californicus* was a 99 % match to the following sequences in GenBank (avian host indicated in parentheses): EU627792 (*Tyto alba*, barn owl), EU627793 (*Strix occidentalis occidentalis*, California spotted owl), KJ488908, and KJ488804 (host species not identified); and KC962151 (*Buteo rufinus*, long-legged buzzard), KC962152 (*Buteo buteo*, common buzzard), HF543622, HF543641, HF543631, HF543617 (*Milvus* spp., kite spp.), KJ577832 (*Larus mongolicus*, Mongolian gull), EF607287 (*Circus aeruginosus*, western marsh harrier), KP000841 (*Buteo buteo*, common buzzard), JX418201 (*Accipiter virgatus*, besra), and EF077166 (*Gavia immer*, common loon). There were no sequences in GenBank that were a 100 % match to the *cyt b* sequence from *L. californicus*.

Phylogenetic analysis

Phylogenetic analysis using Bayesian inference suggests that the *L. californicus* lineage clusters more closely with leucocytozoid lineages found in owls (families Tytonidae and Strigidae) and passerines (families Fringillidae and Emberizidae) than it does with leucocytozoids lineages found in *Buteo* and *Accipiter* (family Accipitridae) (Fig. 1). Of the species included in our analysis, *L. californicus* appears to be most closely related to a *Leucocytozoon* sp. lineage (EU627792) found in the barn owl (*Tyto alba,* Tytonidae), as supported by a posterior probability of 1.0 and a genetic divergence of only 0.44 % (Supplementary Table 2). Genetic divergence in the *cyt b* gene between *L. californicus* and leucocytozoids in diurnal raptors of Accipitridae is relatively large: In *L. buteonis*, found in *Buteo* spp., it ranges between 23.8 and 26.5 %, and in *L. mathisi*, found in *Accipiter* spp., it ranges between 23.0 and 23.3 %. However, a much smaller genetic divergence, ranging from 3.5 to 8.5 %, is seen between the *L. californicus* lineage we recovered in *F. s. sparverius* and the leucocytozoids found in owls and passerines. Divergence between lineages of *L. californicus* and *Leucocytozoon* sp. found in the Tytonidae family is negligible, as presented above.

The *Leucocytozoon* lineage identified in this study was deposited in GenBank™ as accession number KR422359.

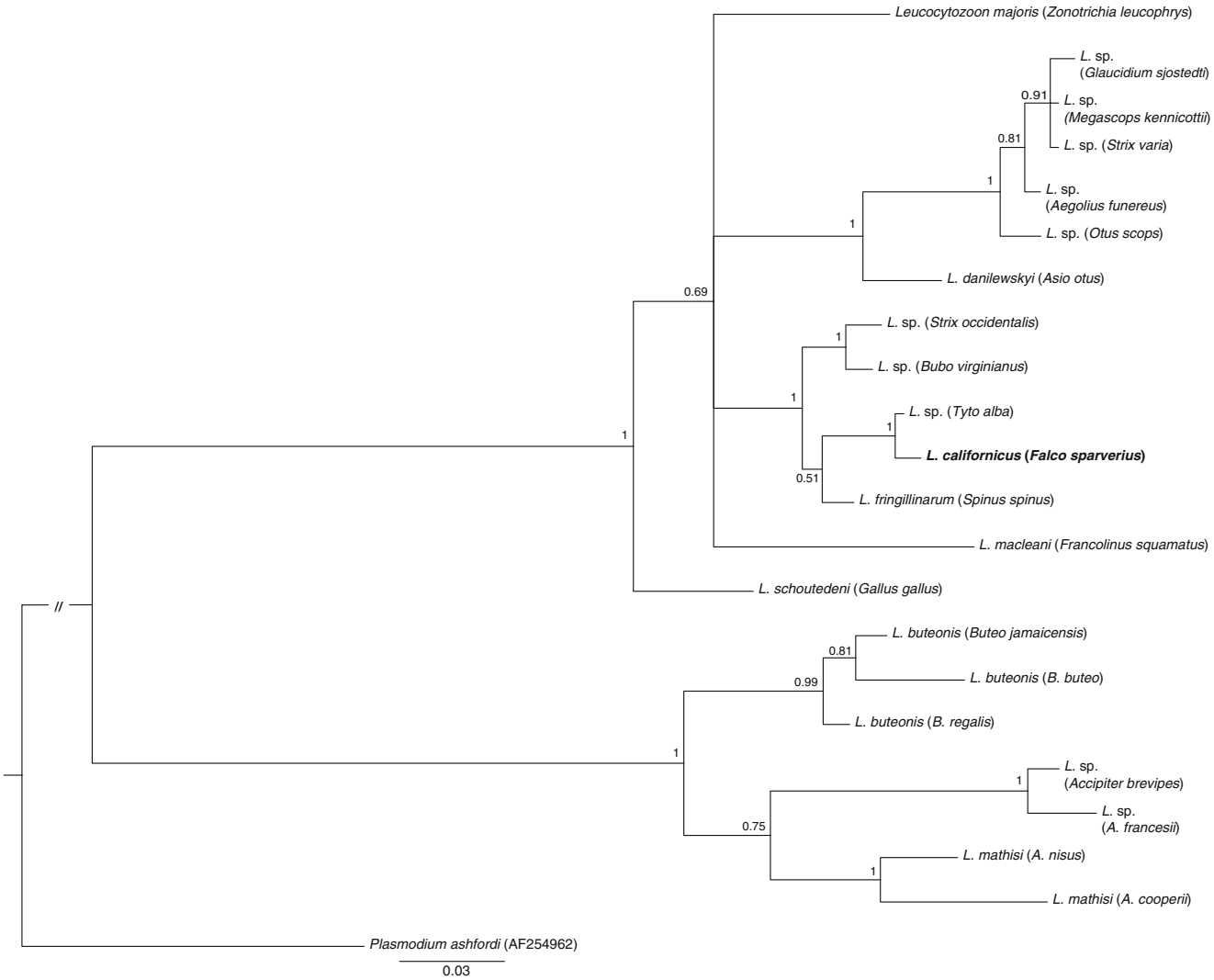

Fig. 1 Bayesian inference phylogeny of 21 mitochondrial cytochrome *b* lineages of *Leucocytozoon* species. *Plasmodium ashfordi* was used as an outgroup. Posterior probabilities of >0.5 are indicated at nodes. Latin names of the avian host are provided in parentheses

Parasite description

Leucocytozoon californicus sp. nov. (Fig. 2, Table 1)

Macrogametocytes (Fig. 2a–d) develop in roundish host cells and are roundish to slightly oval in form. The cytoplasm is heterogeneous in appearance and contains volutin granules and small vacuoles. Parasite nucleus is prominent, variable both in form and in position; nucleolus is visible. Nucleus of host cell is pushed aside, deformed, and lies peripherally as a more or less evident cap or, sometimes, band; it usually extends less than one half the circumference of the gametocyte (Fig. 2a, c) and often assumes a position above the gametocyte (Fig. 2b, d), which is a rare character in avian leucocytozoids. The cytoplasm of host cells is largely replaced by mature gametocytes. The cytoplasm is sometimes invisible but more frequently present around growing gametocytes, and a very pale margin of variable form (Fig. 2c, d).

Microgametocytes (Fig. 2e, f)

The general configuration and other features are as for macrogametocytes with the usual sexual dimorphic characters: the pale staining of the cytoplasm (compare Fig. 2a–f) and the large nuclei of parasites (Table 1).

Taxonomic summary

Type host: Falco sparverius sparverius (Falconiformes, Falconidae).

Additional host: Morphologically indistinguishable gametocytes were reported in the Eurasian hobby (*Falco subbuteo*)

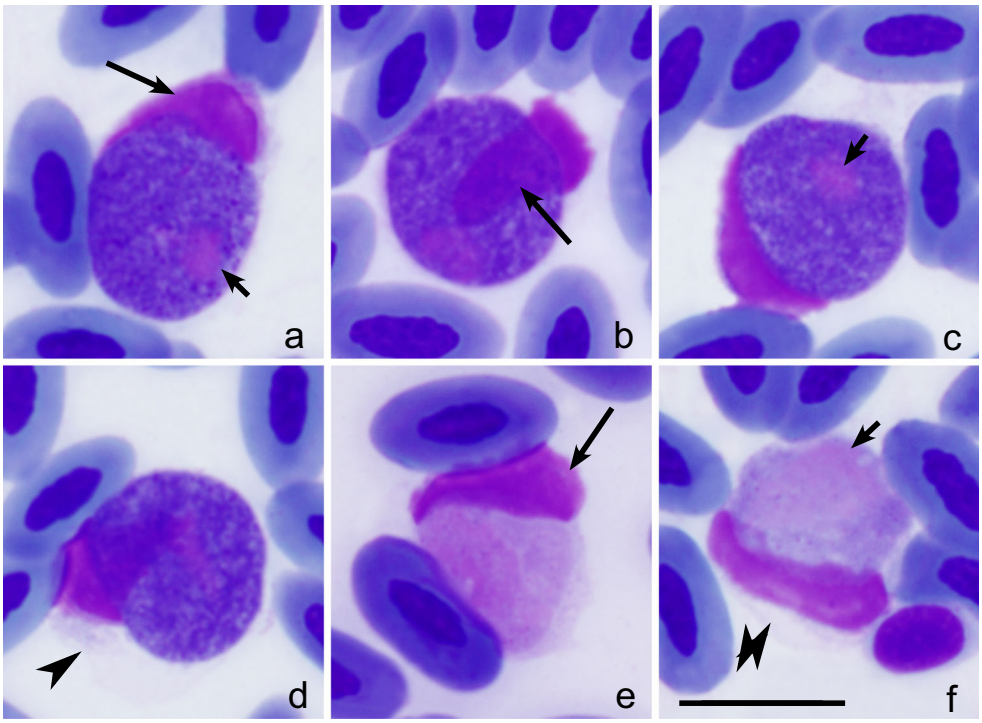

Fig. 2 *Leucocytozoon californicus* sp. nov. (lineage AMKE CA 1 L) from the blood of American kestrel (*Falco sparverius sparverius*): **a**–**d** macrogametocytes, **e**–**f** microgametocytes. Host cell nuclei (*long arrows*), parasite nuclei (*short arrows*), and remnants of the host-cell cytoplasm (*arrowheads*). Note that the host cell nucleus often is located above gametocyte (**b**, **d**), which is rare character in leucocytozoids. Giemsa-stained thin blood films. *Scale bar* = 10 μm

in Southern Kazakhstan (Valkiūnas 1989), but the lineage information is absent.

DNA sequence: GenBank accession KR422359.

Type locality: Mendocino County, California, USA (39.00114–123.07232).

Site of infection: Blood cells; no other data.

Prevalence: Thirteen of 35 (37.1 %) sampled American kestrels were found by PCR to harbor the DNA lineage of *L. californicus*.

Type specimens: Hapantotype (accession number 48830 NS, intensity of parasitemia is approximately 0.002 %, *F. sparverius sparverius*, Mendocino County, CA, USA, 23 May 2012, coll. E. A. Wommack) was deposited in the Institute of Ecology, Vilnius University, Vilnius, Lithuania, and parahapantotype (accession no G465774, intensity of parasitemia is approximately 0.001 %, 19 May 2012, other data as for the hapantotype) was deposited in the Queensland Museum, Queensland, Australia.

Distribution: This parasite has been reported from Mendocino County, California, USA. Reports of morphologically similar parasites in migration falconiform birds in Southern Kazakhstan (Valkiūnas 1989) indicate that transmission likely occurs in Eurasia.

Etymology: The species name reflects the type locality.

Remarks

This is the first species of *Leucocytozoon* described from diurnal raptors in which gametocytes develop exclusively in roundish host cells. All other species of *Leucocytozoon* that parasitize diurnal raptors produce gametocytes that predominantly develop in fusiform host cells possessing more or less evident processes (Greiner and Kocan 1977; Valkiūnas et al. 2010). Gametocytes in roundish host cells have been incidentally and rarely reported in diurnal raptors of the Accipitriformes; gametocytes in fusiform cells are readily predominant (Valkiūnas 2005). For unclear reasons, leucocytozoids are significantly less prevalent and less diverse in Falconiformes than in Accipitriformes (Valkiūnas 2005).

Species of *Leucocytozoon* change the morphology of host cells remarkably, and rapidly, following the very early (trophozoites) stages of development. In all types of host cells, the host cell nucleus is markedly enlarged and deformed, and the amount of cytoplasm is increased (Forrester and Greiner 2008), making it impossible to identify the origin of host cells in *Leucocytozoon* spp. using microscopy of Giemsa-stained blood films. Other histochemistry methods or cell-type-specific antibodies could be applied to clearly identify the origin of host cells of the new species (Zhao et al. 2015). Such analysis would require additional targeted sampling and investigation. Discovery of *L. californicus* opens opportunities for such research in the future.

Based on host range, morphology of host cells, and also the *cyt b* sequence information, the new species can be readily identified. However, the morphology of gametocytes and their host cells observed in *L. californicus* is indistinguishable from many other species of leucocytozoids that develop in roundish host cells, possess cap-like host cell nuclei, and parasitize birds of other avian orders. Thus, sequence information is important for this parasite species identification, as is the case

with the majority of other avian *Leucocytozoon* parasites, because their blood stages possess few morphological features for taxonomic work.

Leucocytozoon franchini, reported by França (1927), has long been considered the only *Leucocytozoon* species described from members of the Falconidae. All other *Leucocytozoon* species parasitizing diurnal raptors were originally described from species of the Accipitridae and Sagittariidae (Greiner and Kocan 1977; Valkiūnas 2005). *Leucocytozoon franchini* has gametocytes in fusiform cells, and it was originally reported in a bird referred to only by its common name, *Albanella pallida*. This bird was formerly thought to belong to the Falconidae; however, it is actually the pale harrier (*Circus macrourus*), a member of the Accipitridae, in which gametocytes of *Leucocytozoon* in fusiform host cells predominate (Valkiūnas 1989). *Leucocytozoon californicus* is readily distinguishable in morphology from *L. franchini.*

Discussion

We found the American kestrels in our study to have a much higher prevalence of *Leucocytozoon* sp. than in previous studies of falcons (Valkiūnas 1989; Korpimaki et al. (1995); Tella et al. 1996; Dawson and Bortolotti 1999; Dawson and Bortolotti 2001). Korpimaki et al. (1995) sampled 160 European kestrel (*Falco tinnunculus*) individuals in western Finland over three breeding seasons and detected a prevalence of around 1 %. Valkiūnas (1989) reported *Leucocytozoon* sp. in 4.7 % of 64 examined falcons in South Kazakhstan. Dawson and Bortolotti (1999, 2001) sampled 442 American kestrel individuals in Saskatchewan over two breeding seasons and found that only one individual was infected with *Leucocytozoon* sp. Tella et al. 1996 sampled 366 lesser kestrels (*Falco naumanni*) in northeastern Spain during two breeding seasons and found no infections of *Leucocytozoon* sp. There are at least three differences between our study, and the studies referenced above that could explain the differing results in *Leucocytozoon* prevalence: (1) The study sites are geographically distant from one another and from our study site; (2) the falcon species sampled in three out of four of the studies differs from the species in our study; and (3) the reference studies utilized only microscopy to detect *Leucocytozoon* spp., whereas our study utilized both PCR and microscopy. Each of these possibilities is addressed here: (1) The variation in study sites could have a significant impact on prevalence due to variation in the suitability of habitat for black flies, the vector for *Leucocytozoon* spp. (Murdock et al. 2013); (2) avian species within the same family can have different susceptibilities to the same parasite species (Valkiūnas 2005; Forrester and Greiner 2008); however, this does not explain the difference in prevalence between our study and that of Dawson and Bortolotti (2001), which also sampled American kestrels; and (3) although some studies have found that microscopy underestimates haemosporidian infection prevalence, these studies evaluated *Plasmodium* and/or *Haemoproteus*, but not *Leucocytozoon* (Fallon and Ricklefs 2008; Jarvi et al. 2002; Richard et al. 2002). Garamszegi (2010) found no significant difference between microscopy and PCR in detecting *Leucocytozoon* (or *Haemoproteus*) infections, and Valkiūnas et al. (2008) demonstrated comparable prevalence results for microscopy and PCR for all three genera but cautioned that successful identification of haemosporidia by microscopy is highly dependent on the quality of blood smears and the skill and experience of the observer.

From a phylogentic standpoint, *L. californicus* appears to be more closely related to *Leucocytozoon* spp. found in birds from the Tytonidae, Strigidae, Fringillidae, Emberizidae, and Phasianidae families, than to *L. buteonis* and *L. mathisi*, species recovered from members of the Accipitridae that share a similar behavioral ecology to *F. sparverius*. While traditional avian phylogenetic studies have placed Falconidae relatively close to Accipitridae (Sibley et al. 1988; Mayr et al. 2003; Livezey and Zusi 2007), several recent studies have found non-monophyly between these two families, suggesting convergence in ecology and morphology (Hackett et al. 2008; McCormack et al. 2013; Jarvis et al. 2014). The analysis of McCormack et al. (2013) of 1500 loci for 32 avian species across as many families placed Falconidae in the same clade as Psittacidae and two passerine families (Viduidae and Pittidae), and relatively far from Tytonidae and Phasianidae. Similarly, Jarvis et al. (2014) placed Falconiform birds close to those in the orders Psittaciformes and Passeriformes, and relatively far from Accipitrifomes and Strigiformes. The analysis of Hackett et al. (2008) placed Falconidae sister to a clade containing Passeriformes and Psittaciformes, whereas Strigiformes are sister to Accipitridae. Our analysis of *Leucocytozoon* species reveals a similar phylogenetic pattern between the blood parasites of Falconiformes and Passeriformes as these three recent studies; however, the close phylogenetic relationship between leucocytozoids of *F. sparverius* and Strigiformes does not correspond to the phylogenetic relationships of their hosts. The lack of perfect correspondence between host and parasite phylogenies may be the result of these parasites' capacities for host-switching in combination with their coevolution, as has been shown in previous work (Ricklefs and Fallon 2002; Ricklefs et al. 2004; Lauron et al. 2014; Ricklefs et al. 2014). We are not aware of any genetic sequence data for *Leucocytozoon* species that have been isolated from Psittaciformes (Martinson et al. 2006, Bensch et al. 2009), although presence of *Leucocytozoon* species has been reported in Psittacidae and Cacatuidae Forrester and Greiner (2008). Additional research is needed to determine which morphospecies of

Leucocytozoon infects species of Psittaciformes, and whether the phylogenetic relationships of the parasite(s) to other leucocytozoids mirrors those of their host's.

Close phylogenetic relationships between *L. californicus* and the parasites of *Tyto alba* were unexpected. In spite of numerous reports of leucocytozoids in this avian host (Bennett et al. 1982), a detailed morphological description of *Leucocytozoon* parasites in *T. alba* is absent. Interestingly, only gametocytes in roundish blood host cells have been reported in *T. alba* in Europe (Valkiūnas 1988, G. Valkiūnas, personal obs.). From this point of view, *L. californicus* and leucocytozoids of *T. alba* are similar and might have evolved due to host switching. Our phylogenetic analysis (Fig. 1) and parasite morphological data (Fig. 2) indicate that *L. californicus* likely infects both *T. alba* (Tytonidae) and *Falco sparverius* (Falconidae). The genetic difference between these parasites is negligible (0.44 %), and gametocytes and their host cells are indistinguishable. It is important to note that gametocytes in fusiform host cells have never been seen in these birds but are common and even predominate in *Leucocytozoon* infections in owls of Strigidae and other birds of prey (Accipitridae; Greiner and Kocan 1977; Bishop and Bennett 1989; Valkiūnas 1989; Peirce et al. 1990; Bennett et al. 1993; Valkiūnas 2005). Because the same species of leucocytozoids usually do not infect nor develop to gametocyte stage, in birds belonging to different orders, this case is important from the point of view of better understanding the vertebrate host specificity in different groups of haemosporidians. Additional experimental studies are needed to prove whether or not *L. californicus* can complete its life cycle in barn and grass owls (Tytonidae).

Leucocytozoon Danilewski parasitizes numerous species of Strigidae all over the world (Ishak et al. 2008) and develops gametocytes both in roundish and fusiform host cells, making it readily distinguishable from *L. californicus*, which is in accord with our phylogenetic analysis. The *L. californicus* and *Leucocytozoon* sp. (from *T. alba*) lineages analyzed in our study have a genetic divergence of <0.5 %, compared to genetic divergences of >20 % between *L. californicus* and both *L. buteonis* and *L. mathisi*, the common parasites of birds of the Accipitridae. Earlier, haemosporidian research has concluded that avian *Plasmodium* and *Haemoproteus* species with a genetic divergence of >5 % in the *cyt b* gene should be morphologically differentiable (Hellgren et al. 2007; Valkiūnas et al. 2009). However, while the former may provide a rule-of-thumb, readily distinguishable morphospecies have been identified for haemosporidian parasites with genetic differentiation of <5 % (Hellgren et al. 2007; Valkiūnas et al. 2009). This underscores the need for careful inclusion of well-established reference species in GenBank that have been reliably identified to morphospecies.

While traditionally understudied compared to *Plasmodium* and *Haemoproteus*, *Leucocytozoon* is increasingly being recognized as a genetically highly diversified genus (Chakarov et al. 2015). While current classifications of *Leucocytozoon* spp. indicate lower morphological diversity relative to *Plasmodium* and *Haemoproteus* (Bensch et al. 2004; Valkiūnas 2005), documented *Leucocytozoon* lineage diversity is likely to increase rapidly with further research combining molecular genetics and morphology (Lotta et al. 2015). An increased understanding of the diversity, distribution, and host species of *Leucocytozoon* spp., combined with ecological data, will facilitate better understanding of the taxonomic structure of this genus, as well as its evolution and effect on raptor populations.

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References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Bennett GF, Whiteway M, Woodworth-Lynas C (1982) A host-parasite catalogue of the avian haematozoa. Mem Univ of Nfld Occ Pap Biol 5:1–243

Bennett GF, Earlé RA, Peirce M (1993) The Leucocytozoidae of South African birds: Falconiformes and Strigiformes. Ostrich 64:67–72

Bensch S, Pérez-Tris J, Waldenström J, Hellgren O (2004) Linkage between nuclear and mitochondrial DNA sequences in Avian Malaria parasites: multiple cases of cryptic speciation? Evolution 58(7): 1617–1621

Bensch S, Hellgren O, Pérez-Tris J (2009) MalAvi: a public database of malaria parasites and related haemosporidians in avian hosts based on mitochondrial cytochrome *b* lineages. Mol Ecol 9:1353–1358

Bishop MA, Bennett GF (1989) The haemoproteids of the avian order Strigiformes. Can J Zool 67:2676–2684

Borner J, Pick C, Thiede J, Kolawole OM, Kingsley MT, Schulze J, Cottontail VM, Wellinghausen N, Schmidt-Chanasit J, Bruchhaus I, Burmester T (2015) Phylogeney of haemosporidian blood parasites revealed by a multi-gene approach. Mol Phylogenet Evol. In press.

Chakarov N, Linke B, Boerner M, Goesmann A, Krüger O, Hoffman JI (2015) Apparent vector-mediated parent-to-offspring transmission in an avian malaria-like parasite. Mol Ecol 6:1355–63. doi:10.1111/mec.13115

Dawson RD, Bortolotti GR (1999) Prevalence and intensity of hematozoan infections in a population of American kestrels. Can J Zool 77: 162–170

Dawson RD, Bortolotti GR (2001) Sex-specific associations between reproductive output and hematozoan parasites of American kestrels. Oecologia 126:193–200

Fallon SM, Ricklefs RE (2008) Parasitemia in PCR-detected *Plasmodium* and *Haemoproteus* infections in birds. J Avian Biol 39:514–522

Forrester DJ, Greiner EC (2008) Leucocytozoonosis. In: Parasitic diseases of wild birds (ed. CT Atkinson, NJ Thomas & DB Hunter), Iowa, Wiley-Blackwell, pp 54–107

França C (1927) Notes Parasitologiques. J Sci Math 5((24):15–24, Fis Nat, Ser. 3

Galtier N, Gouy M, Gautier C (1996) SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. Comput Appl Biosci 12(6):543–548

Garamszegi LZ (2010) The sensitivity of microscopy and PCR-based detection methods affecting estimates of prevalence of blood parasites in birds. J Parasitol 96(6):1197–1203

Greiner EC, Kocan AA (1977) *Leucocytozoon* (Haemosporida; Leucocytozoidae) of the Falconiformes. Can J Zool 55(5):761–770

Hackett SJ, Kimball RT, Reddy S, Bowie RCK, Braun EL, Braun MJ, Chojnowski JL, Cox WA, Han K-L, Harshman J, Huddleston CJ, Marks BD, Miglia KJ, Moore WS, Sheldon FH, Steadman DW, Witt CC, Yuri T (2008) A phylogenomic study of birds reveals their evolutionary history. Science 320:1763–1768

Hellgren O (2005) The occurrence of haemosporidian parasites in the Fennoscandian bluethroat (*Luscinia svecica*) population. J Ornith 146:55–60

Hellgren O, Waldenström J, Peréz-Tris J, Ösi ES, Hasselquist D, Krizanauskiene A, Ottosson U, Bensch S (2007) Detecting shifts of transmission areas in avian blood parasites — a phylogenetic approach. Mol Ecol 16:1281–1290. doi:10.1111/j.1365-294X.2007.03227.x

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogeny. Bioinformatics 17:754–755

Ishak HD, Dumbacher JP, Anderson NL, Keane JJ, Valkiūnas G, Haig SM, Tell LA, Sehgal RNM (2008) Blood Parasites in Owls with conservation implications for the spotted Owl (Strix occidentalis). PLoS One 3(5):E2304

Jarvi SI, Schultz JJ, Atkinson CT (2002) PCR diagnostics underestimate the prevalence of avian malaria (*Plasmodium relictum*) in experimentally-infected passerines. J Parasitol 88:153–158

Jarvis ED et al (2014) Whole-genome analyses resolve early branches in the tree of life of modern birds. Science 346(6215):1320–1331

Korpimaki E, Tolonen P, Bennett GF (1995) Blood parasites, sexual selection and reproductive success of European kestrels. Ecoscience 2:335–343

Krone O, Waldenström J, Valkiūnas G, Lessow O, Müller K, Lezhova TA, Fickel J, Bensch S (2008) Haemosporidian blood parasites in European birds of prey and owls. J Parasitol 94(3):709–715

Lauron EJ, Loiseau C, Bowie RCK, Spicer G, Smith TB, Melo M, Sehgal RNM (2014) Coevolutionary patterns and diversification of avian malaria parasites in African sunbirds (Family Nectariniidae). Parasitol 142:635–647. doi:10.1017/S0031182014001681

Livezey BC, Zusi RL (2007) Higher-order phylogeny of modern birds (Theropoda, Aves: Neornithes) based on comparative anatomy. II. Analysis and discussion. Zool J Linnean Soc 149:1–95

Lotta IA, Gonzalez AD, Pacheco MA, Escalante AA, Valkiūnas G, Moncada LI, Matta NE (2015) *Leucocytozoon pterotenuis* sp. nov. (Haemosporida, Leucocytozoidae): description of the morphologically unique species from the Grallariidae birds, with remarks on the distribution of Leucocytozoon parasites in the Neotropics. Parasitol Res 114(3):1031–44. doi:10.1007/s00436-014-4269-y

Martinson ES, Paperna I, Schall JJ (2006) Morphological versus molecular identification of avian Haemosporidia: an exploration of three species concepts. Parasitol 133:279–288

Mayr G, Manegold A, Johansson US (2003) Monophyletic groups within 'higher land birds' – comparison of morphological and molecular data. J Zool Syst Evol Research 41:233–248

McCormack JE, Harvey MG, Faircloth BC, Crawford NG, Glenn TC, Brumfield RT (2013) A phylogeny of birds based on over 1,500 loci collected by target enrichment and high-throughput sequencing. PLoS One 8(1):e54848

Murdock CC, Foufopoulos J, Simon CP (2013) A transmission model for the ecology of an avian blood parasite in a temperate ecosystem. PLoS One 8:e76126

Nylander JAA, Ronquist JP, Huelsenbeck JP, Nieves-Aldrey JL (2004) Bayesian phylogenetic analysis of combined data. Systematic Biol 53:47–67

Outlaw DC, Ricklefs RE (2011) On the phylogenetic relationship of Haemosporidian parasites from raptorial birds (Falconiformes and Strigiformes). J Parasitol 95(5):1171–1176

Peirce MA, Bennett GF, Bishop M (1990) The haemoproteids of the avian order Falconiformes. J Nat Hist 24:1091–1100

Perkins S, Schall JJ (2002) A molecular phylogeny of malaria parasites recovered from cytochrome *b* sequences. J Parasitol 8:972–978

Raidal SR, Jaensch SM (2000) Central nervous disease and blindness in nankeen kestrels (*Falco cenchroides*) due to a novel *Leucocytozoon*-like infection. Avian Pathology 29:51–56

Raidal SR, Jaensch SM, Ende J (1999) Preliminary report of a parasitic infection of the brain and eyes of a peregrine falcon *Falco peregrinus* and nankeen kestrels *Falco centchroides* in western Australia. Emu 99:291–292

Richard FA, Sehgal RNM, Jones HI, Smith TB (2002) A comparative analysis of PCR-based detection methods for avian malaria. J Parasitol 88(4):819–822

Ricklefs RE, Fallon SM (2002) Diversification and host switching in avian malaria parasites. P R Soc London 269:885–892

Ricklefs RE, Fallon SM, Bermingham E (2004) Evolutionary relationships, cospeciation, and host switching in avian malaria parasites. System Biol 53:111–119

Ricklefs RE, Outlaw DC, Svensson-Coelho M, Medeiros MCI, Ellis VA, Latta S (2014) Species formation by host shifting in avian malaria parasites. PNAS 111:14816–14821

Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574

Sehgal RNM, Hull AC, Anderson NL, Valkiūnas G, Markovets MJ, Kawamura S, Tell LA (2006) Evidence for cryptic speciation of *Leucocytozoon* spp. (Haemosporida, Leucocytozoidae) in diurnal raptors. J Parasitol 92:375–379

Sibley CG, Ahlquist JE, Monroe BL Jr (1988) A classification of the living nirds of the world based on DNA-DNA hybridization studies. Auk 105(3):409–423

Swofford D (2001) *PAUP* 4.0.* Sinauer Associates

Tarello W (2006) *Leucocytozoon toddi* in falcons from Kuwait: epidemiology, clinical signs and response to melarsomine. Parasite 13:97–180

Tella JL, Forero MG, Gajon A, Hiraldo F, Donezar JA (1996) Absence of blood-parasitization effects on lesser kestrel fitness. Auk 113:253–256

Valkiūnas G (1988) Parasitic Protozoa of the blood of birds in the USSR. (3. Leucocytozoidae of Passeriformes, Strigiformes, Anseriformes and Falconiformes) *Lietuvos TSR MA darbai*. C serija 2:114–131 (in Russian)

Valkiūnas G (1989) Occurrence and morphology of two types of *Leucocytozoon toddi* gametocytes in some palearctic Falconiformes. Lietuvos TSR MA darbai. C serija 4(108):46–50 (in Russian)

Valkiūnas G (2005) Avian malaria parasites and other haemosporidia. CRC Press, Boca Raton

Valkiūnas G, Iezhova TA, Križanauskienė A, Palinauskas V, Sehgal RNM, Bensch S (2008) A comparative analysis of microscopy

and PCR-based detection methods for blood parasites. J Parasitol 94:1395–1401

Valkiūnas G, Iezhova TA, Loiseau C, Smith TB, Sehgal RNM (2009) New malaria parasites of the subgenus *Novyella* in African rainforest birds, with remarks on their high prevalence, classification and diagnostics. Parasitol Res 104:1061–1077

Valkiūnas G, Sehgal RNM, Iezhova TA, Hull AC (2010) Identification of *Leucocytozoon toddi* group (Haemosporida, Leucocytozoidae), with remarks on the species taxonomy of leucocytozoids. J Parasitol 96(1):170–177

Zhao W, Liu J, Xu R, Zhang C, Pang Q, Chen X, Liu S, Hong L, Yuan J, Li X, Chen Y, Li J, Su XZ (2015) The gametocytes of Leucocytozoon sabrazesi infect chicken thrombocytes, not other blood cells. PLoS One. Jul 28;10(7):e0133478. doi: 10.1371/journal.pone.0133478. eCollection 2015. Erratum in: PLoS One. 2015;10(8):e0137490.