Parasitol Res (2016) 115:609–613
DOI 10.1007/s00436-015-4777-4

ORIGINAL PAPER

Hepatozoon ellisgreineri n. sp. (Hepatozoidae): description of the first avian apicomplexan blood parasite inhabiting granulocytes

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Received: 2 August 2015 / Accepted: 6 October 2015 / Published online: 16 October 2015

Abstract Blood parasites of the genus *Hepatozoon* (Apicomplexa, Hepatozoidae) infect all groups of terrestrial vertebrates, and particularly high prevalence and species diversity have been reported in reptiles and mammals. A few morphologically similar species, in which gamonts inhabit mononuclear leukocytes and red blood cells, have been described in birds. Here, we report a new *Hepatozoon* species, which was found in wild-caught secretary birds *Sagittarius serpentarius*, from Tanzania. *Hepatozoon ellisgreineri* n. sp. can be readily distinguished from all described species of avian *Hepatozoon* because its gamonts develop only in granulocytes, predominantly in heterophils, a unique characteristic among bird parasites of this genus. Additionally, this is the first reported avian apicomplexan blood parasite, which inhabits and matures in granulocytes. We describe *H. ellisgreineri* based on morphological characteristics of blood stages and their host cells. This finding broadens knowledge about host cells of avian *Hepatozoon* spp. and other avian apicomplexan blood parasites, contributing to the better understanding of the diversity of haematozoa. This is the first report of hepatozoonosis in endangered African birds of the Sagittariidae.

Keywords *Hepatozoon* · New species · Birds · Sagittariidae · Heterophils · Granulocytes

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Introduction

Species of *Hepatozoon* Miller, 1908 (Apicomplexa, Hepatozoidae) are cosmopolitan, arthropod-borne intracellular blood parasites of terrestrial vertebrates (Smith 1996). They have been reported in amphibians and are widespread and diverse in reptiles and mammals, but less diversity has been described in birds (Peirce 2005; Telford 2009; Merino et al. 2014; Ursula et al. 2014; Leal et al. 2015). The complete life cycles are unknown for any of the avian *Hepatozoon* species. However, several life cycles were investigated in reptile and mammal parasites (Smith 1996; Mehlhorn 2008; Telford 2009; Allen et al. 2011; Baneth et al. 2013). Vertebrates usually are infected through ingestion of infected blood-sucking invertebrates (ticks, mites, fleas, bugs and dipteran insects), in which gametogenesis and sporogony occur. Infection via mosquito bite or eating infected vertebrate hosts has been reported in *Hepatozoon* parasites of reptiles (Telford 2009; Viana et al. 2012), but epidemiological significance of these modes of transmission remains insufficiently investigated. Merogony might occur in hepatic parenchymal cells, Kupffer cells, muscle tissues and endothelial cells of capillaries or sinus walls in various organs, preceding the development of gamonts in circulating blood cells. Avian *Hepatozoon* species likely have similar life cycles. Bennett et al. (1992a) reported sporogony of *Hepatozoon atticorae*, the parasite of African cliff swallow *Hirundo spilodera*, in an argasid tick and a flea which are vectors.

Some *Hepatozoon* spp. cause disease in amphibians, reptiles and mammals (Smith 1996; Allen et al. 2011; Telford 2009; O'Dwyer et al. 2011). Little is known about the virulence of *Hepatozoon* parasites in birds. An outbreak of fatal hepatozoonosis in young captive cranes *Grus monacha* was reported in Japan (Shimizu et al. 1987), so avian species of

Hepatozoon may cause disease, which remains insufficiently studied.

Approximately 20 *Hepatozoon* spp. have been described in birds, mainly on the basis of morphological features of their blood stages, which usually inhabit mononuclear leukocytes (Bennett et al. 1992b; Peirce 2005). One described avian *Hepatozoon* species develops in red blood cells (Merino et al. 2014), which are the main host cells for gamonts of *Hepatozoon* parasites in amphibians and reptiles (Smith 1996; Telford 2009). During microscopic examination of blood films obtained from the secretary birds *Sagittarius serpentarius* upon arrival in Denver from Tanzania, we found a morphologically distinct undescribed *Hepatozoon* parasite. Unlike all other avian *Hepatozoon* species, this parasite develops and matures in granulocytes, a unique feature not only in avian *Hepatozoon* spp. but also in the entire group of apicomplexan blood parasites infecting birds. Here, we describe and illustrate this infection and discuss available information about blood parasites reported in the secretary bird, an endangered African bird species.

Materials and methods

Two secretary birds were wild caught in Tanzania and transported to the Denver Zoological Gardens in CO, USA. Their blood was sampled and examined microscopically before addition to the collection at the zoo. The same birds were examined additionally approximately a month after they arrived in the USA. In total, 14 blood films were prepared, air-dried, fixed in absolute methanol and stained with Giemsa. An Olympus BX61 light microscope equipped with Olympus DP70 digital camera and imaging software AnalySIS FIVE was used to examine blood films, to prepare illustrations and to take measurements. Approximately 100–150 fields were examined in blood films at low magnification (400×), and then at least 100 fields were studied at high magnification (1000×). The morphometric features studied (Table 1) are those defined by Bennett et al. (1992b) and Valkiūnas (2005). Intensity of parasitemia was estimated as a percentage by actual counting of the number of parasites per 100 randomly observed granulocytes. The morphometric analysis (Table 1) was carried out using the 'Statistica 7' package.

Results

Microscopic examination revealed *Hepatozoon* parasites in both birds after their capture in Tanzania and when they arrived to the USA. Other blood parasites were not seen. Both infected birds looked healthy.

Table 1 Morphometry of mature gamonts and their host cells of *Hepatozoon ellisgreineri* (n=21)

Feature	Measurements (μm)[a]
Infected heterophils	
Minimum diameter	13.2–15.9 (14.7±0.8)
Maximum diameter	8.2–13.7 (11.3±1.7)
Area	99.1–166.7 (135.7±21.9)
Gamont	
Length	11.8–14.5 (13.4±0.8)
Width	4.9–6.8 (6.0±0.5)
Area	53.5–85.7 (71.0±7.3)
Gamont nucleus	
Length	3.4–7.5 (5.0±0.9)
Width	1.6–5.1 (3.2±0.9)
Area	5.8–20.4 (13.3±4.1)

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

Description

Hepatozoon ellisgreineri n. sp. (Fig. 1a–f, Table 1)

Type host: secretary bird *S. serpentarius* (Accipitriformes, Sagittariidae).

Type locality: Tanzania.

Site of infection: the majority of gamonts (98 %) were present in heterophils; a few parasitized eosinophils were seen. No other data.

Prevalence: overall prevalence was 2 of 2 (100 %).

Distribution: this parasite has been reported only from Tanzania.

Type specimens: hapantotype (accession numbers 48875 NS, intensity of parasitemia is 1.4 % in granulocytes,

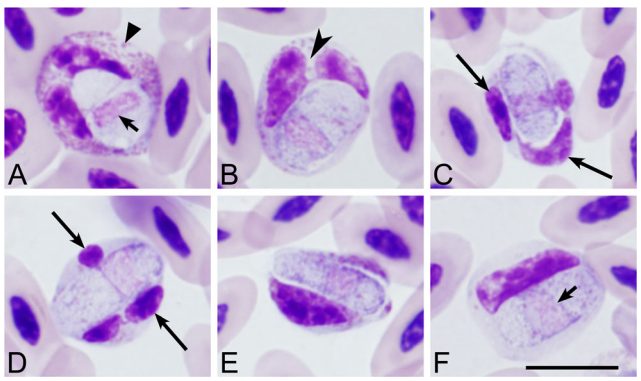

Fig. 1 Growing (**a**–**c**) and fully-grown (**d**–**f**) gamonts of *Hepatozoon ellisgreineri* in the heterophils of the secretary bird *Sagittarius serpentarius*. Giemsa-stained thin films. *Long simple arrows*—nuclei of host cells, *short simple arrows*—nuclei of parasites, *simple arrowhead*—host cell cytoplasm, *triangle arrowhead*—the eosinophilic cytoplasmic granules. Note that the number of the eosinophilic cytoplasmic granules decreases as gamonts mature (compare a–c with d–f). *Scale bar*=10 μm

S. serpentarius, Tanzania, collected by K. Mobley, 2 August 2007) and parahapantotypes (48876, 48877 NS, 3 January 2007, other data as for the hapantotype) were deposited in the Institute of Ecology, Nature Research Centre (NRC), Vilnius, Lithuania. Parahapantotype (accession no. G465775, other data as for the parahapantotype) was deposited in the Queensland Museum, Queensland, Australia. Additional voucher specimens (accessions 48879–48888 NS, data as for the hapantotype and parahapantotypes) were deposited in NRC.

Etymology: this species is named in credit of the prominent parasitologist, Professor Ellis Greiner, in recognition of his contribution to the study of avian blood parasites and initial recognition that this was a novel species.

Gamonts: are associated predominantly with heterophils, but a few gamonts also were seen in eosinophils. Heterophils can be readily distinguished due to (1) the lobed nuclei possessing coarse, clumped, purple-stained chromatin (Fig. 1c, d) and (2) the prominent, colourless or pale-blue stained cytoplasm (Fig. 1c, f) possessing numerous azurophilic granules of various size and shape (Fig. 1a, b, e). The host cell cytoplasm was prominent and well-seen in all parasitized cells (Fig. 1a–f). Number of azurophilic granules markedly decreases in the host cell cytoplasm in the course of gamont maturation: the granules are numerous in the host cells infected with growing parasites (Fig. 1a, b), but are few in the cells with fully-grown organisms (Fig. 1d–f). Gametocytes are broadly elongated with rounded ends, lying close to host-cell nuclear lobes. Cytoplasm of gamonts is uniform, possesses tiny azurophilic granules and lacks visible vacuoles. Parasite nucleus is large (Table 1), usually band or ribbon-like, sometimes appearing as round mass, central or sub-central in position (Fig. 1a, f) and occasionally assumes terminal position. Gamont sex is unapparent.

Discussion

This study reports the first case of *Hepatozoon* parasites in the secretary bird, which is endemic to Africa and the only member of the Sagitariidae. This bird species is still widespread across sub-Saharan Africa, but is assessed as endangered due to the recent rapid population decline across its entire range mainly because of habitat loss and deforestation (BirdLife International 2013). A few studies have addressed blood parasites in this bird species. Until now, only *Haemoproteus* and *Leucocytozoon* sp. have been reported in the secretary bird (Bennett et al. 1982; Bishop, Bennett 1992; Valkiūnas 2005; Atkinson et al. 2008).

The key result of this study is the description of a new avian *Hepatozoon* species, which inhabits granulocytes, and is predominantly reported in heterophils, the highly phagocytic leukocytes which are the most abundant granulocytes in birds (Campbell and Ellis 2007). This is different from what has been reported in other avian *Hepatozoon* species, which parasitize mononuclear leukocytes (Bennett et al. 1992b) and also have been reported in red blood cells (Merino et al. 2014). The cytoplasm is usually hardly visible in mononuclear leukocytes parasitized by all described avian *Hepatozoon* species (Bennett et al. 1992b), but is prominent in all host cells parasitized by *H. ellisgreineri* (Fig. 1a–f). Due to these features of host cells, this infection can be readily distinguished from all other infections caused by avian *Hepatozoon* parasites (Bennett et al. 1992b; Peirce 2005; Merino et al. 2014). Additionally, fully-grown gamonts of *H. ellisgreineri* are large (Table 1), and they can be comparable only with *Hepatozoon albatrossi*, based on this character. Average length of gamonts in all other described avian *Hepatozoon* species does not exceed 11 μm (Bennett et al. 1992b; Merino et al. 2014).

Hepatozoon species often inhabit neutrophils in mammals (Smith 1996; Mehlhorn 2008; Baneth et al. 2013). Avian heterophils are functionally equivalent to the mammalian neutrophils. These leukocytes actively participate in controlling various infections, including parasitic ones, being an essential part of the innate immune system (Campbell and Ellis 2007). Both the heterophils infected with *H. ellisgreineri* and those not infected looked similar: the nuclei of the majority of host cells were lobed and possessed readily visible clumps of chromatin, and the cytoplasm usually appeared colourless and possessed eosinophilic granules. Interestingly, numbers of the cytoplasmic granules markedly decreases in the course of maturation of gamonts (compare Fig. 1a, b, d–f). Because the eosinophilic granules of heterophils contain enzymes involved in phagocytic process (Hodges 1977; Campbell and Ellis 2007), it seems possible that one of the mechanisms responsible for the parasite survival in these phagocytes is the reduction of the cytoplasmic granules during maturation of gamonts. Further studies are needed for better understanding of this process.

Subsequent examinations of blood films from infected secretary birds revealed no parasites approximately 2 years after discovery of this infection. We lack samples for molecular characterization of *H. ellisgreineri* and re-sampling is not possible. Only two studies addressed molecular characterization and phylogeny of avian *Hepatozoon* species, both using partial sequences of the 18S rRNA. Merino et al. (2014) supported *Hepatozoon peircei*, the parasite inhabiting red blood cells of the storm petrel *Oceanodroma melania*, belonging to the genus *Hepatozoon*, but relationships of this infection to hepatozoids inhabiting other vertebrates remained unclear. Biedrzycka et al. (2013) believed that *Hepatozoon*-like parasites reported in the serge warbler *Acrocephalus schoenobaenus* are most likely *Lankesterella* sp. However, because the latter study also detected sequences of *Caryospora* and *Eimeria* from the same bird population using the same set of primers (Hep800F/Hep1615R), it is difficult to rule out an opportunity of co-infection of *Hepatozoon* sp. with *Lankesterella* sp. and resulting preferable amplification of

DNA of the latter parasite instead of *Hepatozoon* sp.; additional studies are needed to test this conclusion. This is particularly worth considering because PCR-based diagnostics using general primers often does not read co-infections of closely related apicomplexan parasites belonging to different genera due to preferable amplification of parasite DNA, to which the primers do a better match (Valkiūnas et al. 2006; Martínez et al. 2009; Braga et al. 2011; Dimitrov et al. 2015). Because DNA of *Caryospora* and *Eimeria* species was amplified from the blood samples (Biedrzycka et al. 2013), the used primers certainly are insufficiently specific for detection of *Hepatozoon* spp. This problem in PCR-based diagnostics of blood parasites is sensitive in wildlife (Valkiūnas et al. 2014), but remains insufficiently addressed. Additional molecular studies using multigene analysis and phylogenies based on DNA sequences obtained using specific primers are needed for better understanding of the taxonomic position of avian *Hepatozoon* sp.

It is worth noting that *H. ellisgreineri* is the first described bird apicomplexan blood parasite, which parasitizes and matures exclusively in granulocytes. Other described avian apicomplexan blood parasites have not been reported to mature in granulocytes probably due to their high immunologic activity in birds (Levine 1988; Smith 1996; Valkiūnas 2005; Campbell and Ellis 2007; Atkinson et al. 2008). Merozoites of *Leucocytozoon simondi* were seen in heterophils and eosinophils, but gametocytes probably do not develop (Desser et al. 1970). Development in granulocytes has not been reported in *Hepatozoon* species parasitizing amphibians or reptiles, but some mammalian inhabiting species are present in neutrophils (Smith 1996; Telford 2009; Ursula et al. 2014; Leal et al. 2015). This study shows that diversity of avian *Hepatozoon* is greater than has been assumed. Additional research is needed for better understanding these bird pathogens. The obstacles for avian *Hepatozoon* research are (1) the insufficient knowledge about diseases caused in birds; (2) the light parasitemia, which can be readily overlooked; (3) the lack of good morphological criteria for separation of species and (4) the lack of data about full life cycles of all described species, resulting in absence of information about parasite host specificity. Unfortunately, the sensitive PCR-based diagnostics alone is insufficient in studies of life cycles of apicomplexan parasites because it does not distinguish different stages of life cycle and does not read abortive parasite development, which may be a result of parasite presence but without potential for completing the life cycle (Martínez et al. 2009; Valkiūnas et al. 2014). Experimental studies aimed at understanding the patterns of development of *Hepatozoon* spp. in avian hosts combined with PCR-based and microscopic methods are needed, but such studies are still absent in these widespread bird parasites.

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