Experimental Parasitology 145 (2014) 74–79

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Haemoproteus minutus and *Haemoproteus belopolskyi* (Haemoproteidae): Complete sporogony in the biting midge *Culicoides impunctatus* (Ceratopogonidae), with implications on epidemiology of haemoproteosis

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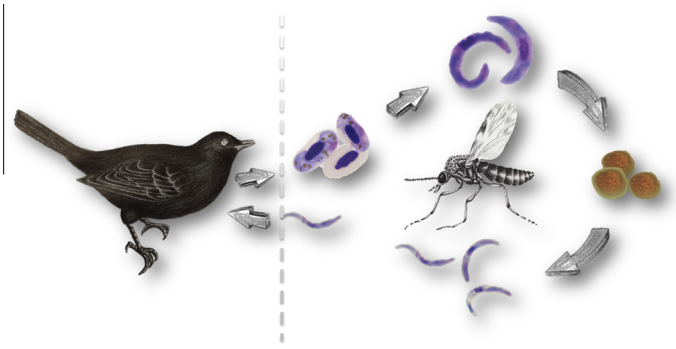
HIGHLIGHTS

- Deadly *Haemoproteus minutus* is transmitted by *Culicoides impunctatus*.
- Sporogony of *H. belopolskyi* completes in *C. impunctatus*.
- Sporogonic stages of *H. minutus* and *H. belopolskyi* are described and illustrated.
- Ookinetes and sporozoites of these parasites are readily distinguishable morphologically.
- *Culicoides impunctatus* is an important vector of *Haemoproteus* parasites.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 31 March 2014
Received in revised form 21 July 2014
Accepted 25 July 2014
Available online 4 August 2014

Keywords:
Haemoproteus
Haemosporidian parasite
Sporogony
Culicoides
Transmission
Epidemiology

ABSTRACT

Species of *Haemoproteus* (Haemoproteidae) are cosmopolitan haemosporidian parasites, some of which cause severe diseases in birds. Numerous recent studies address molecular characterization, distribution and genetic diversity of haemoproteids. However, the information about their vectors is scarce. We investigated sporogonic development of two widespread species of *Haemoproteus* (*Haemoproteus minutus* and *Haemoproteus belopolskyi*) in the experimentally infected biting midge *Culicoides impunctatus*. Wild-caught flies were allowed to take blood meals on naturally infected common blackbirds *Turdus merula* and icterine warblers *Hippolais icterina* harboring mature gametocytes of *H. minutus* (lineage hTURDUS2) and *H. belopolskyi* (hHIICT1), respectively. The engorged flies were collected, transported to the laboratory, held at 15–18 °C, and dissected daily in order to obtain ookinetes, oocysts and sporozoites. Mature ookinetes of *H. minutus* developed blisteringly rapidly; they were numerous in the midgut content between 1 and 4 h post exposure. Ookinetes of *H. belopolskyi* developed slower and were reported 1 day post exposure (dpe). Oocysts of both parasites were seen in the midgut wall 3–4 dpe. Sporozoites of *H. minutus* and *H. belopolskyi* were first observed in the salivary glands preparations 7 dpe. The percentage of experimentally infected flies with sporozoites of *H. minutus* was 82.1% and 91.7% with *H. belopolskyi*. In accordance with microscopy data, polymerase chain reaction amplification and sequencing confirmed presence of the corresponding parasite lineages in experimentally infected biting midges. Sporogonic stages of these parasites were described and illustrated. This study indicates that *C. impunctatus* is involved in the transmission of deadly *H. minutus*, which kills captive parrots in Europe. This biting midge is an important vector of avian haemoproteids and worth more attention in epidemiology research of avian haemoproteosis.

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http://dx.doi.org/10.1016/j.exppara.2014.07.014
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1. Introduction

Species of *Haemoproteus* (Haemosporida, Haemoproteidae) are widespread in birds in countries with temperate and tropical climates (Valkiūnas, 2005; Atkinson, 2008). Numerous recent studies addressed molecular characterization, distribution and genetic diversity of haemoproteids. However, few studies deal with vectors and transmission of avian *Haemoproteus* spp. (Atkinson, 1991; Desser and Bennett, 1993; Valkiūnas et al., 2002; Martínez-de la Puente et al., 2011; Santiago-Alarcon et al., 2012; Levin et al., 2012). Biting midges of *Culicoides* (Diptera, Ceratopogonidae) and louse flies (Hippoboscidae) transmit these parasites, but certain vector species remain unknown for the great majority of avian haemoproteids and their lineages (Atkinson, 2008; Clark et al., 2014).

Avian *Haemoproteus* spp. have been traditionally considered relatively benign in their avian hosts (Bennett et al., 1993). However, numerous field studies (Nordling et al., 1998; Merino et al., 2000; Møller and Nielsen, 2007; Valkiūnas, 2005; Atkinson, 2008) and limited experimental observations (Atkinson et al., 1988; Marzal et al., 2005; Valkiūnas et al., 2006) indicate negative influence of these parasites on bird fleetness. Several species of *Haemoproteus* have been reported to cause diseases, sometimes even lethal in non-adapted birds (Miltgen et al., 1981; Atkinson et al., 1988; Cardona et al., 2002; Ferrell et al., 2007; Donovan et al., 2008; Olias et al., 2011; Cannell et al., 2013). However, the true extent of pathology and mortality caused by *Haemoproteus* parasites remains unclear because the severe haemoproteosis and death of infected birds occur mainly during the tissue stage of parasite development, before the appearance of parasitemia (Cannell et al., 2013). Such diseases are difficult to diagnose both by microscopic and polymerase chain reaction (PCR)-based diagnostic methods. It is important to note that *Haemoproteus* infections are virulent in some vectors and even kills bird-biting dipteran insects (Levin and Parker, 2014; Valkiūnas et al., 2014). These parasites are worthy of more attention in veterinary medicine, epidemiology and conservation biology studies.

Recent PCR-based findings indicate that *Haemoproteus minutus* is responsible for some instances of mortality in captive parrots in Europe (Olias et al., 2011; Palinauskas et al., 2013). This parasite is widespread and relatively benign in common blackbirds *Turdus merula* in Europe, but it kills several species of captive parrots on the stage of megalomeronts, if these birds are exposed to the infection. Vectors of *H. minutus* parasite remain unknown.

The sporogony of only few *Haemoproteus* spp. has been investigated in detail in biting midges (Linley, 1985; Valkiūnas, 2005; Santiago-Alarcon et al., 2012); it is difficult to work with these insects due to their tiny size and difficulties to colonize the majority of their species (Miltgen et al., 1981; Valkiūnas, 2005; Atkinson, 2008). It was shown that *Culicoides impunctatus* transmits several species of haemoproteids in Europe (Valkiūnas, 2005). This biting midge was reported as a vector of *Haemoproteus belopolskyi* from blackcaps *Sylvia atricapilla* (Valkiūnas and Iezhova, 2004). However, recent molecular studies show that this blackcap parasite is actually *Haemoproteus parabelopolskyi* (Valkiūnas et al., 2007), and the vector of *H. belopolskyi*, which parasitize icterine warblers *Hippolais icterina* needs to be identified.

Because studies on vectors and transmission of avian *Haemoproteus* spp. are uncommon and vectors of *H. minutus* and *H. belopolskyi* are unknown, the aim of this study was to follow sporogony of these parasites in the biting midge *C. impunctatus*, which is widespread in Europe, willingly takes blood meal on birds and is susceptible to several haemoproteid infections (Glukhova, 1989; Blackwell, 1997; Valkiūnas, 2005).

2. Materials and methods

2.1. Study site, collection of blood samples and experimental birds

This study was carried out at the Biological Station of the Zoological Institute of the Russian Academy of Sciences on the Curonian Spit in the Baltic Sea (55°09′ N, 20°52′ E) between 21 May and 2 July in 2013. The experiments described herein comply with the current laws of Lithuania and Russia. Experimental procedures of this study were approved by the International Research Co-operation Agreement between the Biological Station Rybachy of the Zoological Institute of the Russian Academy of Sciences and Institute of Ecology of Nature Research Centre (25-05-2010). All efforts were made to minimize handling time and potential suffering of birds. None of the experimental birds suffered apparent injury during experiments.

Birds were captured with mist nets and identified. About 30 μl of blood was collected in heparinized microcapillaries by puncturing the brachial vein and stored in SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0) at ambient temperature while in the field, and then preserved at −20 °C in the laboratory. A drop of blood was taken from each bird to make two or three blood films. The smears were air-dried, fixed in absolute methanol and stained with Giemsa, as described by Valkiūnas et al. (2008). Blood films were prepared and examined microscopically before each exposure of flies in order to check the level of parasitemia and to detect any other possible relapsed infections. Intensity of parasitemia was estimated as a percentage by counting the number of mature gametocytes per 1000 erythrocytes examined. The species of *Haemoproteus* were identified according to Valkiūnas (2005). Naturally infected birds with single infections were used as donors to infect biting midges. Blood samples from donor birds were examined for haemosporidian parasites by PCR amplification. Positive amplifications were sequenced and cytochrome *b* (cyt *b*) lineages of *Haemoproteus* parasites were determined in the laboratory (see description below).

Two common blackbirds *T. merula* naturally infected with *H. minutus* (lineage hTURDUS2, parasitemia 0.1%) and two icterine warblers *H. icterina* infected with *H. belopolskyi* (lineage hHIICT1, parasitemia 0.2%) were used as donors of gametocytes to infect biting midges. One uninfected juvenile common crossbill *Loxia curvirostra* was used to feed a control group of flies. All birds were kept indoors in a vector-free room under controlled conditions [55–60% relative humidity (RH), 20 ± 1 °C, the natural light–dark photoperiod (L/D) 17:7 h]; they were fed standard diets for seed eating or insectivorous bird species. All birds survived to the end of this study and were released after experimental work.

2.2. Wild-caught biting midges

Experimental infection of biting midges *C. impunctatus* with two *Haemoproteus* species was performed near Lake Chaika, located close to the village of Rybachy, where density of the flies was high (Glukhova and Valkiūnas, 1993; Liutkevičius, 2000; Valkiūnas et al., 2002). To minimize the probability of natural infection of wild-caught midges with *Haemoproteus*, the first generation of naturally occurring flies was used in this study. All experimental infections were performed between 10 and 20 June when the first generation of *C. impunctatus* predominated (Liutkevičius, 2000). Unfed flies were collected by entomological net at this study site before experiments. Some were fixed in 70% ethanol and used for morphological species identification, and the remainder were fixed in 96% ethanol and used for PCR-based identification and determining natural prevalence of *Haemoproteus* infection (see description below).

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