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Haemoproteus tartakovskyi (Haemoproteidae): Complete sporogony in *Culicoides nubeculosus* (Ceratopogonidae), with implications for avian haemoproteid experimental research

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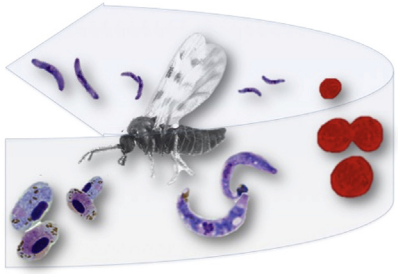
HIGHLIGHTS

- Sporogony of *Haemoproteus tartakovskyi* completes in *Culicoides nubeculosus*.
- Sporogonic stages of *H. tartakovskyi* were described and illustrated.
- *Culicoides nubeculosus* is a good experimental vector of *Haemoproteus* spp.

GRAPHICAL ABSTRACT



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ABSTRACT

Numerous recent studies have addressed the molecular characterization, distribution and genetic diversity of *Haemoproteus* spp. (Haemoproteidae). Some species of these blood parasites cause severe disease in birds, and heavy infections are often lethal in biting midges (Ceratopogonidae) and other blood-sucking insects. However, information about the vectors of haemoproteids is scarce. This presents an obstacle for better understanding the mechanisms of host–parasite interactions and the epidemiology of haemoproteosis. Here we investigated the sporogonic development of *Haemoproteus tartakovskyi*, a widespread bird parasite, in experimentally infected biting midges, *Culicoides nubeculosus*. These biting midges are widespread in the Europe. The insects were cultivated under laboratory conditions. Unfed females were allowed to take blood meals on wild caught siskins *Carduelis spinus* naturally infected with *H. tartakovskyi* (lineage hSISKIN1). Engorged females were maintained at 22–23 °C, dissected at intervals, and examined for sporogonic stages. Mature ookinetes of *H. tartakovskyi* were seen in the midgut content between 6 and 48 h post infection, oocysts were observed in the midgut wall 3–4 days post infection (dpi). Sporozoites were first reported in the salivary gland preparations 7 dpi. In accordance with microscopy data, polymerase chain reaction amplification and sequencing confirmed presence of the corresponding parasite lineage in experimentally infected biting midges. This study indicates that *C. nubeculosus* willingly takes blood meals on birds and is a vector of *H. tartakovskyi*. These biting midges are readily amenable to cultivation under laboratory conditions. *Culicoides nubeculosus* transmits *Haemoproteus* parasites infecting parrots, owls and siskins, birds belonging to different families

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and orders. Thus, this vector provides a convenient model for experimental research with avian haemoproteids.

1. Introduction

Numerous recent studies have addressed the molecular characterization, distribution and genetic diversity of haemoproteids (Haemosporida, Haemoproteidae) (Atkinson, 1991; Desser and Bennett, 1993; Santiago-Alarcón et al., 2012; Bobeva et al., 2013; Pérez-Rodriguez et al., 2015). However, few studies have identified the vectors and their role in the transmission of avian *Haemoproteus* spp. (Valkiūnas, 2005; Santiago-Alarcón et al., 2012; Žiegytė et al., 2014). This is particularly true at the level of the numerous genetic lineages of these parasites, whose competent vectors remain unknown (Atkinson, 1991; Desser and Bennett, 1993; Valkiūnas et al., 2002; Martinez-de la Puente et al., 2011; Santiago-Alarcón et al., 2012). Many recent studies have reported the molecular detection of haemosporidian parasites in wild-caught blood-sucking dipteran insects (Kim et al., 2009; Njabo et al., 2009; Kimura et al., 2010; Martinez-de la Puente et al., 2011; Ferrraguti et al., 2013; Synek et al., 2013; Tanigawa et al., 2013; Bobeva et al., 2015). This method readily indicates significant links between infected vertebrate hosts and blood-sucking insects. However, it does not distinguish among stages of parasite development, including the abortive parasite development in blood-sucking insects (Valkiūnas et al., 2014a). The observation of sporozoites remains essential in confirming insects as vectors.

Species of *Haemoproteus* are widespread in birds throughout the world (Valkiūnas, 2005; Atkinson, 2008), including countries with cold climates (Oakgrove et al., 2014). Approximately 150 species of avian haemoproteids have been described (Iezhova et al., 2011). They are markedly diverse, and some species have been reported to cause severe and even lethal pathologies in bird species (Miltgen et al., 1981; Atkinson et al., 1988; Olias et al., 2011; Križanauskienė et al., 2013; Cannell et al., 2013). However, the veterinary significance of haemoproteosis remains insufficiently understood because parasites might damage organs of birds before the development of parasitaemia (Valkiūnas et al., 2014b). Blood-sucking dipteran insects belonging to the families Ceratopogonidae and Hippoboscidae are vectors of these parasites (Bennett et al., 1982; Atkinson, 2008; Santiago-Alarcón et al., 2012).

Knowledge of the patterns of sporogonic development of hemoproteids in blood-sucking dipterans is important because these parasites are virulent not only to birds, but also to their vectors (Levin and Parker, 2014; Valkiūnas et al., 2014a). Moreover, a heavy *Haemoproteus* spp. parasitaemia can kill bird-biting dipterans (Valkiūnas and Iezhova, 2004; Valkiūnas et al., 2014a).

Experimental and field studies on *Culicoides* species transmitting *Haemoproteus* parasites are scarce mainly because these insects are small, and difficult to infect and maintain in the laboratory (Atkinson, 1991; Valkiūnas, 2005; Žiegytė et al., 2014). Methodologies of maintaining colonies of only a few species of these insects have been developed (Boorman, 1974). It is difficult to design and perform experimental studies with wild-caught biting midges (Valkiūnas, 2005; Atkinson, 2008), which results in a paucity of information about *Haemoproteus* spp. vectors. *Culicoides impunctatus* biting midges transmit several *Haemoproteus* species in Europe (Valkiūnas et al., 2002; Santiago-Alarcón et al., 2012; Žiegytė et al., 2014). Wild-caught *C. impunctatus* and laboratory cultivated *Culicoides nubeculosus* have been used in the experimental research of avian haemoproteids so far (Valkiūnas, 2005; Žiegytė et al., 2014; Bukauskaitė et al., 2015). *Culicoides nubeculosus* biting midges transmit *Haemoproteus handai* (the parasite of parrots) , *Haemoproteus noctuae* and *Haemoproteus syrnii* (the parasites of owls) (Miltgen et al., 1981; Bukauskaitė et al., 2015), but there is no information about the ability of this insect to transmit haemoproteids of passeriform birds.

Culicoides nubeculosus is the largest midge in the *Culicoides* genus (Gutsevich, 1973). It is the only European Culicoides species, of which colonies have been successfully maintained at laboratory conditions (Boorman, 1974). This colony is available at the Pirbright Institute (Pirbright, UK). For these reasons, this insect is a convenient model organism in studying the sporogonic development and parasite–vector relationships of *Haemoproteus* spp. Recent study by Bukauskaitė et al. (2015) showed that two *Haemoproteus* species complete their sporogonic development in *C. nubeculosus*, however, it remains unclear how many other species of avian haemoproteids can be transmitted by this biting midge. The aim of this study was to investigate and describe sporogonic development of *Haemoproteus tartakovskyi,* a widespread parasite of passeriform birds, in experimentally infected biting midges *C. nubeculosus.* This study enhances the knowledge of *Haemoproteus* parasite vectors and encourages other researchers to use *C. nubeculosus* as a model organism in studies of vector–parasite interactions.

2. Materials and methods

2.1. Collection and examination of bird blood samples

We used *H. tartakovskyi* (the lineage hSISKIN1, GenBank accession number KF754352), which was maintained in naturally infected siskins *Carduelis spinus* on the Curonian Spit in the Baltic Sea (55°09′ N, 20°52′ E) between 25 of May and 1 of July, 2014. Birds were caught with mist nets and identified. About 30 μl of blood was collected in heparinized microcapillaries by puncturing the brachial vein for molecular analysis. The samples were stored in SET-buffer (Hellgren et al., 2004) at ambient temperature in the field and then preserved at −20° C in the laboratory. A drop of blood was taken from birds to make two blood films, which were air-dried, fixed in absolute methanol and stained with Giemsa as described by Valkiūnas et al. (2008). An Olympus BX–43 light microscope equipped with Olympus SZX2–FOF digital camera and imaging software “QCapture Pro 6.0, Image-Pro Plius” was used to examine preparations and prepare illustrations. Approximately 100–150 fields were examined at low magnification (× 400), and then at least 100 fields were studied at high magnification (× 1000) in order to evaluate the parasitaemia. Intensity of parasitaemia was estimated as a percentage by actual counting of the number of parasites per 1000 erythrocytes or per 10 000 erythrocytes if infections were light (<0.1%), as recommended by Godfrey et al. (1987). Species of *Haemoproteus* parasites were identified according to Valkiūnas (2005). Two naturally infected birds with single infection of *H. tartakovskyi* were used as donors to infect biting midges. No other avian blood parasites were detected in the donor bird, as confirmed by extensive microscopic examination and PCR-based testing (see description below).

2.2. Experimental design

Biting midges were reared in the P.B. Šivickis Laboratory of Parasitology according to Boorman (1974). The methodology of cultivation is similar to the cultivation of Culicidae mosquitoes. Briefly, larvae were reared in plastic dishes; they were fed with purchased grass meal and ground wheat germ. Mature pupae were collected and placed in small (approximately 5 cm in diameter) cardboard boxes covered with fine mesh bolting silk. Adult insects were maintained in these boxes, and they were infected experimentally according to a protocol described by Kazlauskienė et al. (2013). The insects were allowed to take blood meals on legs of siskins harbouring mature gametocytes of *H. tartakovskyi* (lineage SISKIN1). One colony of biting midges was released in cages ($12 \times 12 \times 12$ cm^3), which were made of fine-mesh bolting silk. A zip faster was sewn into one wall of the cage to permit entry of the bird's legs, which were inserted into an insect cage, and biting midges were allowed to take blood meals on the legs. Engorged insects flew off the bird's legs into the insect cage, which was then closed. Biting midges willingly took blood meals on legs of bird, and the majority of females were fully engorged approximately 20 min after the exposure. The engorged insects were maintained in the laboratory at 22–23° C, 60% RH, L/D photoperiod of 17:7 h, supplied with 5–10% saccharose solution. The insects were dissected daily in order to follow the development of ookinetes, oocysts and sporozoites of the parasite.

2.3. Dissection of biting midges and making preparations of ookinetes, oocysts and sporozoites

In all, 39 individual *C. nubeculosus* females were infected and dissected for analysis of ookinetes (8 midges), oocysts (9) and sporozoites (22). The engorged biting midges were dissected daily in a drop of 0.85% saline. They were lightly anesthetized by putting them into a tube closed with a cotton pad wetted in 96% ethanol for several minutes (Kazlauskienė et al., 2013). Wings and legs of the insects were removed before dissection, which was performed under the binocular stereoscopic microscope Olympus SZ X 10. Preparations of ookinetes, oocysts and sporozoites were prepared according to Valkiūnas (2005). Midgut content was examined for ookinetes, the midgut wall – for oocysts, and the salivary glands – for sporozoites. Smears of midgut contents and salivary glands were dried in the air, fixed with methanol, stained with Giemsa, and examined in the same way as blood films. In order to make permanent preparations of oocysts the midgut was removed from the infected females, fixed in 10% formalin, stained with Ehrlich's hematoxylin and mounted in Canada balsam, as described by Kazlauskienė et al. (2013). After making the preparations of ookinetes and sporozoites, the remains of the same biting-midge were preserved individually in 96% ethanol for PCR-based detection of the parasite. At least 2 samples of each parasite developmental stage were analysed to confirm identity of the parasite lineage. We used a new dissecting needle and a new microscope slide for each dissected biting midge in order to eliminate contamination of samples.

2.4. Microscopic examination of vector preparations

All preparations were examined with Olympus BX–43 light microscope equipped with Olympus SZX2–FOF digital camera; the imaging software "QCapture Pro 6.0, Image-Pro Plius" was used to examine preparations, prepare illustrations and to take measurements. All vector preparations were first examined at low magnification (× 600) and then at high magnification (× 1000). Images of ookinetes, oocysts and sporozoites were collected for measurements from 3 to 5 preparations of different infected biting midges. The morphometric analysis (Table 1) was carried out using the "Statistica 7" package.

Representative preparations of ookinetes (accessions number 48889 NS), oocysts (48890 NS) and sporozoites (48891–48892 NS) were deposited in the Nature Research Centre, Vilnius, Lithuania.

2.5. Extraction of DNA, polymerase chain reaction and sequencing

Blood samples from donor birds as well as infected biting midges were examined for haemosporidian parasites by PCR amplification. Total DNA was extracted from bird blood and individual biting-midges using ammonium acetate DNA extraction method (Richardson et al., 2001). Cytochrome *b* (cyt *b*) gene fragment was amplified using nested PCR protocol (Bensch et al., 2000; Hellgren et al., 2004). Two pairs of initial primers, HaemNFI and HaemNR3 were used for detection of *Haemoproteus*, *Plasmodium* and *Leucocytozoon* species. For the second PCR, we used primers HAEMF and HAEMR2, which are specific to *Haemoproteus* and *Plasmodium* parasites. All samples were evaluated by running 1.5 μl of PCR product on a 2% agarose gel. One negative control (nuclease free water) and one positive control (an infected sample, which was confirmed as positive by microscopic examination of blood films) were used per every 8 samples. No cases of false positive were reported. The sequences were edited using BioEdit program (Hall, 1999) and aligned in BLAST at NCBI (National Centre of Biotechnology Information website: http//www.ncbi.nlm.nih.gov/BLAST) to determine lineages of detected DNA sequences. Possible presence of haemosporidian co-infections in donor birds was ruled out due to absence of "double bases" in electropherograms.

2.6. Ethical statement

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 the experimental birds. All experimental birds survived to the end of this study.

Table 1
Morphometric parameters of ookinetes, oocysts and sporozoites of *Haemoproteus tartakovskyi* (genetic lineage hSISKIN1) in *Culicoides nubeculosus* biting midges.

Feature	*H. tartakovskyi*
Ookinete	
Length	15.8–23.2 (20 ± 1.6)
Width	1.4–3.4 (2.3 ± 0.6)
Area	22–50.1 (32.9 ± 9)
Oocyst	
Minimum diameter	4.8–9.8 (7.2 ± 1.9)
Maximum diameter	5.6–12.4 (9 ± 2.2)
Area	21.2–93 (53.3 ± 27.2)
Sporozoite	
Length	6.3–10.1 (8.7 ± 0.9)
Width	0.8–1.5 (1.1 ± 0.2)
Area	7.6–12.2 (9.6 ± 1.2)

[a]Measurements of ookinetes (n = 21, methanol-fixed preparations 1 day post infection, dpi), oocysts (n = 15, formalin-fixed preparations of mature parasites 3–4 dpi) and sporozoites (n = 21, methanol-fixed preparations 8 dpi) are given in micrometres. Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation.

3. Results

According to both microscopic examination (Fig. 1a, b) and PCR-based testing, infections of solely *H. tartakovskyi* were present in two siskins used as donors to infect biting midges. Intensity of parasitaemia of mature gametocytes was approximately 0.1% in both donor birds prior to infection. PCR-based analysis proved that both donor birds were infected with the lineage hSISKIN1 of *H. tartakovskyi*. This parasites completed sporogony in the experimentally infected *C. nubeculosus* biting midges.

Numerous zygotes and ookinetes were seen in the midgut contents of the exposed midges between 6 h post infection (hpi) and 2 days post infection (dpi) (Fig. 1c–e, Table 1). Ookinetes were elongated wormlike bodies with prominent centrally located nuclei. Readily visible "vacuoles" and pigment granules were present in the cytoplasm of growing and mature ookinetes. Oocysts were seen in the midgut of all 9 infected individuals 3–4 dpi (Fig. 1f, Table 1). In the mercurochrome stained preparations, the oocysts appeared as small roundish bodies with readily visible wall and pigment granules. Up to 7 oocysts were observed in midgut

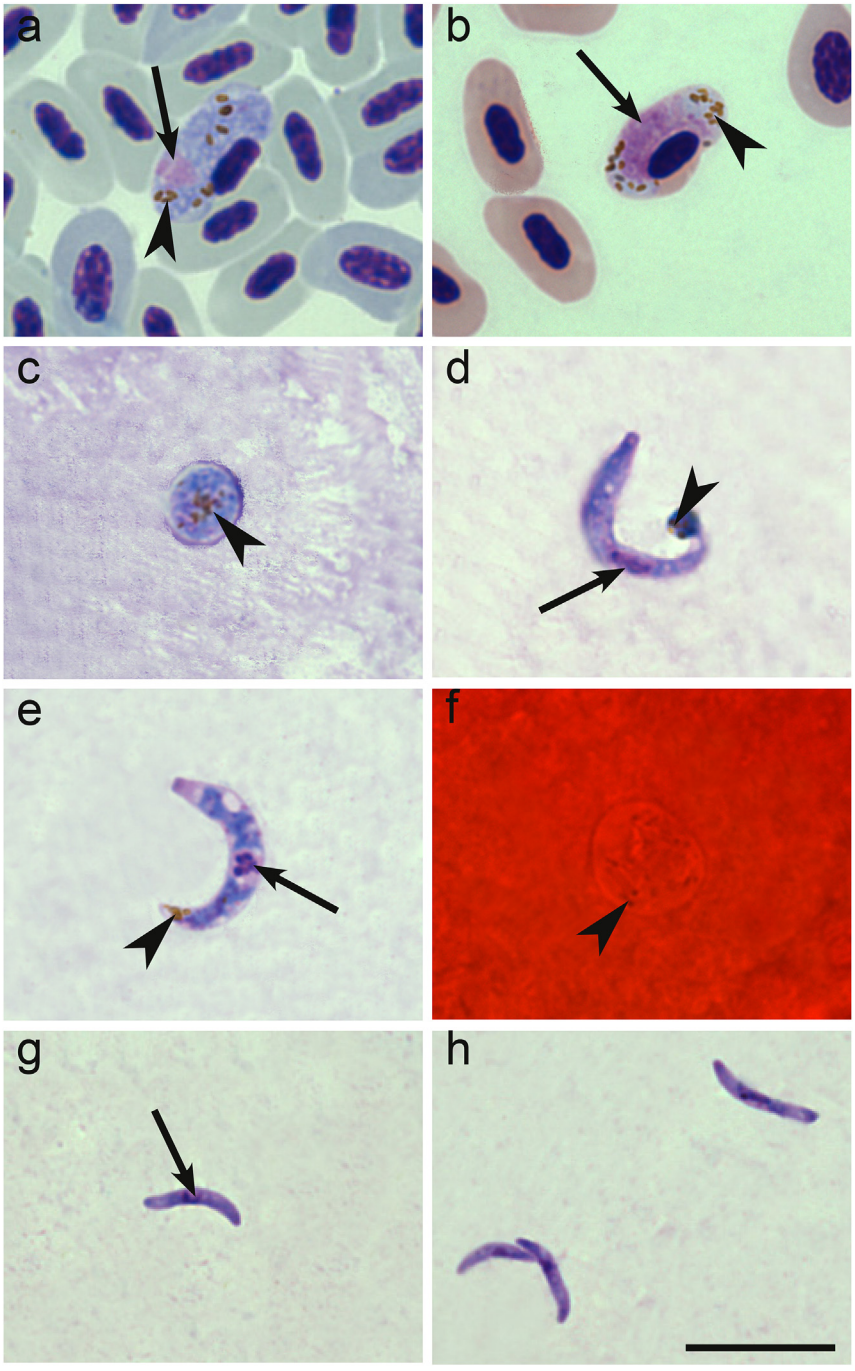

Fig. 1. Gametocytes (a–b) and sporogonic stages (c–h) of *Haemoproteus tartakovskyi*: mature macrogametocyte (a) and microgametocyte (b) in the peripheral blood of the siskin *Carduelis spinus*. Zygote (c), growing okinete (d), mature ookinete (e), oocyst (f), sporozoites (g, h) in the experimentally infected biting midge *Culicoides nubeculosus*. Methanol-fixed and Giemsa-stained thin films (a–e, g, h). Formalin-fixed whole mounts stained with Erlich's hematoxylin (f). Long simple arrows – nuclei of parasites, arrowheads – pigment granules, Scale bar = 10 μm.

preparations, but their true number is difficult to calculate in permanent preparations due to the tiny size of the oocysts, which often are masked by non-digested blood and midgut tissues. Sporogony completed, and sporozoites were observed in the salivary glands of biting midges 7–11 dpi; they were seen in salivary gland preparations of all dissected insects (Fig. 1g–h). Sporozoites were fusiform bodies with centrally located nuclei and approximately equally pointed ends (Fig. 1g–h).

In accordance with microscopy data, PCR-based methods confirmed the presence of the lineage hSISKIN1 in experimentally infected biting midges.

4. Discussion

The key result of this study is that the widespread parasite *H. tartakovskyi* (the lineage hSISKIN1) completes sporogony and produces sporozoites in *C. nubeculosus* biting midges (Fig. 1g, h). Miltgen et al. (1981) and Bukauskaitė et al. (2015) reported the complete sporogony of *Haemoproteus* parasites of parrots and owls in the same species of biting midges, respectively. This study adds *C. nubeculosus* to the list of vectors of haemoproteids parasitizing birds belonging to Passeriformes, indicating that this biting midge serves as a vector of avian haemoproteids parasitizing birds of different families and even orders, including Psittaciformes and Strigiformes.

Culicoides nubeculosus is widely distributed in the Western Palaearctic, and it is particularly abundant in mixed forest zone (Gutsevich, 1973; Bobeva et al., 2013). It is the only European *Culicoides* species that can be successfully maintained in laboratory conditions (Boorman, 1974; Pages et al., 2014). It is convenient for experimental research not only in the transmission of Bluetongue virus in sheep (Pages et al., 2014) but also of avian haemoproteids (Miltgen et al., 1981; Bukauskaitė et al., 2015; this study). It is known that *C. nubeculosus* midges feed on mammals (Jennings and Mellor, 1988), but previous studies (Miltgen et al., 1981; Bukauskaitė et al., 2015) and our experience indicate that they also willingly feed on birds blood in captivity. Larvae and pupae of *C. nubeculosus* are mostly found in organic matter-rich breeding sites (Uslu and Dik, 2006).

The investigated parasite, *H. tartakovskyi,* is widespread in passerine birds in the Palaearctic. The type vertebrate host of this parasite is the crossbill *Loxia curvirostra* (Valkiūnas, 2005). Additional vertebrate hosts are the hawfinch *Coccothraustes coccothraustes* and the siskin (Passeriformes).

It is worth noting that *H. tartakovskyi* (hSISKIN1) has been recently used as a model organism in experimental and evolutionary biology studies. This parasite is a convenient for investigation of *in vitro* development of haemoproteids (Valkiūnas et al., 2014c). It was successfully used for the extraction and purification of large amounts of DNA material from microgametes; which has proven important for haemosporidian genomic research (Palinauskas et al., 2013). The first genome of avian haemoproteid *H. tartakovskyi* was recently sequenced and corresponding publication has been submitted to press (S. Bensch personal communication). Heavy parasitaemia of this parasite causes mortality in blood-sucking mosquitoes due to the damage of insect tissues by migrating ookinetes (Valkiūnas et al., 2014a). Due to these features, *H. tartakovskyi* deserves more attention, and the identification of its potential vectors provides new opportunities for experimental research aiming better understanding this pathogen.

According to available data, *H. tartakovskyi* completes sporogony and produces sporozoites in two species of biting midges: *C. nubeculosus* (this study) and *C. impunctatus* (Valkiūnas et al., 2002). In the latter insect, ookinetes of *H. tartakovskyi* were observed in midguts 36 hpi. This study showed that ookinetes are visible in *C. nubeculosus* between 6 and 48 hpi. Oocysts were observed in the midgut wall of both insect species from 3 dpi. Interestingly, sporozoites were seen in the salivary glands of *C. impunctatus* from 5 dpi, and in *C. nubeculosus* – from 7 dpi. However, because thermal conditions were different in these two experimental studies, additional research is needed to better understand differences in rates of sporogonic development of haemoproteids in different species of biting midges.

The abundance of *C. impunctatus* imago is high only once a year (from the end of May till the end of – June) at our study site (Liutkevičius, 2000; Žiegytė et al., 2014), thus this insect species can be used for experiments only during a short period of a year in wild. Additionally, biting midges of this species are difficult to maintain at laboratory conditions, and they have never been colonised. This hinders the use of *C. impunctatus* in experimental vector studies. *Culicoides nubeculosus* can be readily grown in colonies and used for continuous experiments (Boorman, 1974; Bukauskaitė et al., 2015), and we recommend using this insect in experimental and epidemiology studies of avian haemoproteids.

PCR-based diagnostics alone are insufficient in proving vector competence of the parasite, so experimental research remains of great importance in determining the vectors of avian haemoproteids. PCR-based methods are however helpful in determining possible vectors by identifying blood meal preferences of insects (Kazlauskienė et al., 2013; Bobeva et al., 2015). Recent studies based on PCR show that *Haemoproteus* spp. DNA can be found not only in *Culicoides* spp. (Santiago-Alarcón et al., 2013; Bobeva et al., 2015), but also in many species of blood sucking mosquitoes (Culicidae) (Santiago-Alarcón et al., 2013; Ferraguti et al., 2013; Synek et al., 2013), which are not vectors of *Haemoproteus* parasites. Certainly, experimental studies combining microscopy and PCR-based methods are essential in haemosporidian vector research.

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