

The use of nest boxes in determination of biting midges involved in transmission of *Haemoproteus* parasites

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Short report

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Abstract

Background

Culicoides biting midges (Diptera, Ceratopogonidae) are known to be vectors of avian Haemoproteus parasites. These parasites cause disease and pathology in birds. The diversity of biting midges in Europe is great, but only four Culicoides species are known to be vectors of avian Haemoproteus parasites. In general, our knowledge about the role of the particular Culicoides species in transmission of Haemoproteus parasites remains insufficient. Information gaps hinder a better understanding of parasite biology and the epizootiology of parasite-caused diseases. The aim of this study was to determine new ornithophilic Culicoides species potentially involved in local transmission of Haemoproteus parasites. To do this we collected biting midges in bird nest boxes, identified their species and prevalence of haemoproteids in insects as well as in juvenile birds during breeding season.

Methods

Biting midges were collected from bird nest boxes, identified and parous females were tested individually for the presence of haemoproteids. The blood of juvenile birds was sampled to determine a local transmission of Haemoproteus spp. in the study area. We have used both microscopy and PCR-based methods.

Results

In all, 293 Culicoides females belonging to 11 species were collected from nest boxes. Culicoides pictipennis, Culicoides segnis and Culicoides kibunensis were determined as dominant species collected using this method. Culicoides kibunensis was found to be infected with Haemoproteus lanii (genetic lineage hRB1), C.pictipennis and Culicoides punctatus- with Haemoproteus minutus (hTUPHI01 and hTURDUS2, respectively), C.segnis- with Haemoproteus majoris (hCWT4), H.minutus (hTURDUS2) and Haemoproteus tartakovskyi (hSISKIN1). From 187 studied juvenile birds 31 were infected with H. majoris (hCWT4, hPARUS1, hPHSIB1, hWW2) and Haemoproteus sp. (hPARUS10) parasites, which are widespread in Europe.

Conclusions

Our results provided information about the distribution of biting midge species and determined new ornithophilic Culicoides species at the study site. This study contributes to epizootiology of avian Haemoproteus infections by specifying Culicoides species that likely are responsible for the transmission of haemoproteids in Europe. Used method is suitable for better understanding vector ecology and evaluating the role of different blood sucking insects in transmission of haemoproteids in different wild ecosystems.

Background

Blood-sucking biting midges (Ceratopogonidae: Culicoides) play an important role in various wildlife processes, they are transmitters of viruses, bacteria, parasitic protozoa and nematodes [1–3]. They are vectors of the Haemoproteus (Haemosporida) parasites, which can cause diseases and even lethal pathology in non-adapted birds [1–5]. At present there are 1368 species of biting midges known in the world [6], but only 12 of them have been proved to support complete sporogony of avian Haemoproteus parasites [7, 8], though about 150 species of Haemoproteus have been described [9]. *Culicoides impunctatus* is one of the most abundant species in North Europe [11, 12], therefore the most exhaustive experimental studies on sporogony of haemoproteids have been done with wild *C. impunctatus* biting midges [2, 10]. Other experimental studies were performed with *Culicoides nubeculosus*, which is the only Palearctic *Culicoides* species cultivated in laboratories [7, 13]. Recently, Bernotienė et al., [8] detected *Haemoproteus* sporozoites in wild-caught *Culicoides kibunensis* biting midges using both microscopy and PCR-based methods. Another study describing sporogonic development of haemoproteids till the infective stage (sporozoites) in *Culicoides downesi*, *Culicoides bottimeri*, *Culicoides sphangnumensis*, *Culicoides edeni*, *Culicoides hinmani*, *Culicoides arboricola*, *Culicoides haematopotus*, *Culicoides knowltoni*, *Culicoides stilobezziodes* and *Culicoides crepuscularis* was published by Atkinson et al. [3]. However, most of these biting midge species except *C. sphangnumensis* are distributed in North America and cannot be found in Europe. Several recent studies have reported other *Culicoides* species as possible *Haemoproteus* vectors, but their analyses were based only on molecular determination of the parasite DNA obtained from wild-caught insects [14–18]. PCR-based studies are helpful in determination of ornithophilic biting midges [19] and this information is essential in vector research. Presence of parasite's DNA indicates that the insect might be possible vector, but these data alone are insufficient to prove the transmission of haemoproteids, because PCR-based diagnostics cannot distinguish between invasive for vertebrate hosts (sporozoites) and non-invasive sporogonic stages [20]. Our knowledge about the composition of *Culicoides* species, which may be involved in transmission of *Haemoproteus* parasites in wild is insufficient and it interferes for better understanding patterns of epizootiology of vector-borne diseases in wildlife [2, 3, 21]. Therefore, the aim of this study was to identify ornithophilic biting midges obtained from bird nest boxes and to determine which species of *Culicoides* could potentially participate in the transmission of *Haemoproteus* parasites in wildlife.

Birds, the intermediate hosts of *Haemoproteus* parasites, are the most vulnerable to bites of *Culicoides* midges during the nesting and juvenile care period [2]. This short and vulnerable for the host period was used in our study. First, we have collected midges from nest boxes as described by Tomas et al. [22]. Biting midges of these species were intentionally looking for the bird blood, so were ornithophilic and could potentially play a role in transmission network of *Haemoproteus* parasites. Second, to find out if caught biting midges contained DNA of *Haemoproteus* spp., we applied PCR-based analysis for each of collected insect. Third, to determine, which haemosporidian parasites are transmitted at study areas we checked juvenile birds (they can be infected only at the study site) for the presence of *Haemoproteus* parasites during breeding period. This allowed us to indicate potential vectors of avian *Haemoproteus* parasites and to plan further experimental studies on sporogony of haemoproteids in biting midges of new species.

Materials And Methods

Study site

This study was carried out at the Neris Regional park (NRP), Lithuania (54°50'N 24°58'E) and at the Biological Station Rybachy (BSR) of the Zoological Institute of the Russian Academy of Sciences on the Curonian Spit located of the Baltic Sea (55°15'N, 20°86'E). Studies of biting midges were carried out in May–July during 2012, 2018 and 2019 (BSR) and in 2017 (NRP), juvenile birds were investigated in June–August during 2016, 2018, 2019 (BSR).

Collection of biting midges and microscopic examination

Blood-sucking insects were collected according to methodology described by Tomas et al. [22]. Nest boxes were attached to the tree up to 2 meters high (Fig. 1 a). Petri dishes moisten with baby oil were temporarily fixed upside down using double sided sticky tape on inside roofs of nest boxes (Fig. 1 c). Insects flying inside the nest boxes stuck to the Petri dishes, thus could be collected (Fig. d). Seventy three nest boxes with nesting birds and hatched nestlings were investigated: eleven in NRP and sixty-two in BSR (14, 28 and 20 in 2012, 2018 and 2019, respectively).

Petri dishes were left overnight and were removed next day, because *Culicoides* biting midges are active at dusk [12]. Petri dishes were replaced several times per week from the end of May to the end of July. Petri dishes with sticking insects were taken to the laboratory, biting midges were anesthetized with 96% ethanol, identified according to the morphological features [23–25]. The heads of biting midges were removed to prepare permanent preparations mounted in Euparal for the further identification of *Culicoides* species.

Parous females were detected according to the presence of the readily visible burgundy pigment in the subcutaneous cells of the abdomen, indicating a digested blood meal prior to capture [26]. These females were dissected for preparation of salivary glands as described by [2, 27]. Invasive stage of haemoproteids (sporozoites) can be found in salivary glands of infected biting midges [2]. Briefly, insects were placed in a small drop of 0.9 % normal saline. Head and thorax were separated. Extracted salivary glands were grinded using a needle and mixed with a tiny drop of saline. Preparations were dried in the air, fixed with absolute methanol, and stained with 4% Giemsa stain. Remnants of insects were placed for PCR-based confirmation of the insect species and the detection of parasite's DNA in insect using primers for haemosporidian parasites (as described below) [28–30]. To eliminate contamination of samples, we used a new dissecting needle for each dissected biting midge. All material was studied under the binocular stereoscopic microscope Olympus SZX10 and Olympus BX43 microscope (Tokyo, Japan).

Collection of juvenile bird blood and microscopic examination

In order to detect haemoproteids which are transmitted in the study area, the juvenile birds of the species which usually nesting in nest boxes (*Parus major*, *Poecile palustris*, *Cyanistes caeruleus*, *Ficedula hypoleuca*) were captured by mist nets during fieldwork. About 50 µl of blood was taken in heparinized microcapillaries by puncturing the brachial vein. A small drop was used for preparation of two blood smears from each individual. Residual blood was stored in SET-buffer for molecular analysis [31]. The smears were air-dried, fixed in absolute methanol and stained with Giemsa stain solution, as described by [32]. Approximately 100–150 fields were examined at low magnification (400×), and then at least 100 fields were studied at high magnification (1000×). The intensity of parasitemia 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. [33]. We used an Olympus BX43 light microscope (Tokyo, Japan) to analyze the blood slides. *Haemoproteus* parasites were identified according to Valkiūnas [2]. All birds were released in the same area as captured after blood sampling.

Polymerase chain reaction and sequencing

Total DNA was extracted from each individual biting midge and from the bird blood using ammonium acetate DNA extraction method [34].

For detection of haemoproteids the segment of parasite mitochondrial cytochrome b (*cyt b*) gene fragment was amplified using nested PCR protocol [29, 30]. The initial primers HaemNFI and HaemNR3 and nested primers HaemF and HaemR2 were used.

To confirm identification of *Culicoides* species we used insect specific primers LC0149 and HCO2198 to amplify a fragment of cytochrome c oxidase subunit 1 (*cox1*) of mitochondrial DNA [28]. Morphological identification was consistent with PCR-based identification of biting midges.

Fragments of DNA of all PCR positive samples were visualized on 2% agarose gel using MidoriGreen dye (NIPPON Genetics Europe, Germany). All positive samples were sequenced using corresponding primers and sequences were edited and aligned using BioEdit program [35]. Mixed infections of parasites were determined by the visualization of double-base calling in sequence electropherograms. All positive for *Haemoproteus* spp. biting midge samples were double checked with multiplex PCR [36] primers, which amplify the fragment between the 5' end of *cyt b* and a non-coding region of mtDNA which is outside of HaemF/HaemR2 fragment. The genetic analyzer “Basic Local Alignment Search Tool” (National Centre of Biotechnology Information website: <http://www.ncbi.nlm.nih.gov/BLAST>) was used to determine lineages of detected parasite and insect sequences.

Results

Biting midge species and prevalence of *Haemoproteus* parasites in midges obtained from nest boxes

In all, 293 females of *Culicoides* biting midges were collected and studied: 127 of them were collected in the NRP, 166 in BSR, 64 (2012), 79 (2018) and 23 (2019) (Table 1).

Ten *Culicoides* species and *Culicoides obsoletus* species group were determined between collected individuals in nest boxes. *Culicoides kibunensis* was the most abundant species in NRP (52 % of all sampled midges), while *Culicoides segnis* and *Culicoides pictipennis* were dominant in BSR (37,5% and 74,7 % of all sampled midges, respectively in 2012 and 2018) (Table 1). The abundance of biting midges was low in 2019 in spite of the fact that the timing and the same sampling method was used (Table 1). This year the most abundant species (30,4%) was *C. kibunensis*.

Biting midges in bird nest boxes were collected from the end of May until the end of July. The biggest number of collected biting midges in one nest box was determined in NRP. Up to 48 biting midges on the 1st of June in one nest box were collected. The number of collected biting midges in BSR was smaller. Up to 22–24 biting midges were collected in one nest box on the 30th of May in 2018, up to 9 biting midges were collected on the 12th of June in 2012. Only up to 4 biting midges were collected in one nest box on the 6th of June in 2019.

Five genetic lineages of *Haemoproteus* parasites were detected using PCR based methods in biting midges belonging to four *Culicoides* species (Table 1): *C. kibunensis* (1 female), *C. pictipennis* (5 females), *C. segnis* (3 females), *C. punctatus* (1 female). No infected biting midges were collected in 2019. Two *C. kibunensis* and one *C. reconditus* contained DNA of *Plasmodium* parasites, *P. homonucleophilum* (genetic lineage pSW2) and *P. relictum* (pSGS1) respectively (Table 1).

Microscopic examination of preparations from salivary glands of PCR positive biting midges were negative.

Parasites in juvenile birds

In all 187 juvenile birds belonging to species *Cyanistes caeruleus*, *Parus majoris*, *Poecile palustris*, *Ficedula hypoleuca*, were captured and analyzed: 2016 (14 birds), 2018 (100 birds) and 2019 (73 birds). Presence of a single infection was confirmed after sequencing parasite's DNA by visual inspection of double-base-calling on the electropherogram [37] and microscopic examination of blood slides.

Using both PCR and microscopy we have detected, that 31 out of 187 investigated juvenile birds were infected with haemoproteids. Juvenile birds were infected with different genetic lineages of *Haemoproteus majoris* (genetic lineages: hCWT4, hPARUS1, hPHSIB1, hWW2,) and *Haemoproteus* sp. (hPARUS10) at the study site before leaving for their wintering grounds (Table 2).

Discussion

It is known that sporogony of different *Haemoproteus* species can take place in four European *Culicoides* species: *C. impunctatus*, *C. nubeculosus*, *C. kibunensis* and *C. sphagnumensis*. *Culicoides impunctatus* is one of the most abundant species of *Culicoides* in North Europe as well as in our study site [12, 38]. This species is excellent experimental vector and likely is natural vector of 12 species of *Haemoproteus* parasites [10]. Biting midges of this *Culicoides* species are extremely abundant in June in some localities and this allows using them in experimental research [2, 10]. *Culicoides impunctatus* used to be considered as mammalophilic species [39], but cases of ornithophilic behavior of these biting midges have been documented [10]. Our study confirms ornithophilic *C. impunctatus* behavior as this insect was found visiting nest boxes of breeding birds in our study.

Culicoides kibunensis was detected as a vector of *Haemoproteus pallidus* in Lithuania, because two wild caught individuals of this species were detected to harbor DNA as well as sporozoites of *H. pallidus* (lineage hPCF1) [8]. *Culicoides nubeculosus* is the only Palearctic *Culicoides* species cultivated in laboratory, that is why, experimental studies on sporogony of several *Haemoproteus* spp. in these biting midges were performed in recent years [7, 13]. Some studies followed sporogony of haemoproteids till the sporozoite stage in *C. sphagnumensis* [3]. The diversity of *Culicoides* in the Europe is high – more than 100 species are known [25] and there is no information about other *Culicoides* species which would be known as vectors of *Haemoproteus* parasites and would be involved in the transmission at our study site. Experimental studies of parasite sporogony in vectors are difficult to design, because of the complexity and work load. To conduct such experiments several components are crucial 1) donor bird with a single chronic infection, 2) high abundance of blood sucking insects, 3) facilities for keeping engorged insects, 4) dissection of midguts and salivary glands from these tiny insects. But first of all, we have to know, which *Culicoides* species willingly take blood meal from birds and can be used in the experiment as a potential vectors of bird haemoproteids. Information about host preference and possible vector species should help to plan more detailed experimental studies of sporogony process using microscopy and PCR-based methods.

Birds, at nesting time are easy targets for blood sucking insects [2], so the collection of insects in bird nest boxes can help both to determine ornithophilic insect species and to identify infected biting midges. The host range of biting midges is difficult to determine and it remains insufficiently investigated. Additional data providing information about host preference of *Culicoides* biting midges are important for epizootiology studies. Five out of 11 *Culicoides* species, collected in nest boxes, have been already known to take blood meals on birds: *Culicoides kibunensis*, *C. pictipennis*, *C. segnis*, *C. impunctatus* and *C. festivipennis* [10, 15–17, 21, 40], but only sporadic cases of ornithophily have been reported for biting midges belonging to other species [40]. *Culicoides reconditus*, *C. subfascipennis*, *C. pallidicornis*, *C. punctatus* and *C. obsoletus* were also collected in nest boxes thus they likely naturally were looking for bird blood in the wild.

Culicoides obsoletus and *C. punctatus* are among the most abundant biting midges in North Europe [41, 42], thus they should be considered for experimental research as potential vector candidates for *Haemoproteus* transmission. *Culicoides kibunensis*, *C. segnis* and *C. pictipennis* being the dominant species attacking birds, as determined in this study, were not known to be abundant at study site. It was documented that *C. impunctatus* was the most dominant species in the Curonian spit and formed 82.1 – 99.7 % of all *Culicoides* [12, 43, 44] and this species is still the dominant in some localities of the Curonian spit in June. *Culicoides* species being the dominant in nest boxes according to our data were even not detected using other collection methods (light, netting, collection from humans) on the Curonian spit during earlier investigations [12, 43]. The method applied for insect collection may have crucial impact on species composition and abundance of collected insects, this method to collect biting midges from nest boxes may be of great importance with the target to find ornithophilic species and potential vectors of avian haemoproteids.

PCR-based testing of wild-caught insects for the presence of *Haemoproteus* DNA can also be helpful in detecting potential vectors of avian haemoproteids, but this method alone is insufficient to demonstrate that the insect is a vector of the parasite [19]. Experimental studies indicate that avian malaria parasites can persist even in resistant blood sucking insects for several weeks after initial blood meals due to the survival of ookinetes. These parasites can be gained only during infected blood meals on birds, so the presence of parasite's DNA proves only that biting midge have taken blood meal from the bird before [20]. According to PCR-based testing, nine *Culicoides* species are known to harbor *Haemoproteus* parasite DNA in Europe. These are *Culicoides alazanicus* [15], *Culicoides circumscriptus*, *C. festivipennis* [8, 15, 45], *C. kibunensis* [8, 16, 17], *C. pictipennis* [8, 17, 45], *C. segnis* [16], *C. scoticus* [8, 17], *C. punctatus* and *C. obsoletus* [8]. We have detected avian haemosporidian parasites in biting midges belonging to 5 *Culicoides* species and have added *C. reconditus* to this list (Table 1).

Parasites of *Haemoproteus majoris* (hCWT4), *Haemoproteus minutus* (hTURDUS2) and *Haemoproteus tartakovskiyi* (hSISKIN1) were found in three *C. segnis* females (Table 1). Four genetic lineages of haemoproteids (hCUKI1, hTUPHI01, hCCF4 and hROFI1) have been detected in *Culicoides segnis* biting midges in Europe by Synek et al. [16], so we have supplemented information about *Haemoproteus* parasites detected in this *Culicoides* species. Based on these data, it is likely, that *C. segnis* could be a potentially new vector of some haemoproteids. For confirmation of vector status, detailed experiments of sporogony should be performed using this biting midge species in the future.

Culicoides punctatus and *C. pictipennis* females were found in nest boxes and were infected with *H. minutus* (hTURDUS2 and hTUPHI01, respectively) (Table 1). These parasites are widespread in common blackbirds *Turdus merula* in Europe and in our study site [46]. *Haemoproteus minutus* cause mortality in captive parrots in Europe by causing lethal disease on the stage of megalomeronts [45, 47]. It was shown that *H. minutus* (hTURDUS2) can be transmitted by *C. impunctatus* [27] and laboratory cultivated *C. nubeculosus* biting midges [7]. Known data indicate broad susceptibility of the *C. impunctatus* and *C. nubeculosus* biting midges to many *Haemoproteus* parasites [7, 10] and in general shows low vector specificity of the haemoproteids, but *C. nubeculosus* has been not detected at our study site and the

distribution of *C. impunctatus* is very sporadic, so other *Culicoides* species should be involved in the transmission of different *Haemoproteus* parasites.

The presence of *Plasmodium* DNA reported in *C. kibunensis* and *C. reconditus* during this study, show preferability of these biting midges to feed on birds [19], but not possible transmission of *Plasmodium* parasites [2]: *Culicoides* biting midges do not transmit avian *Plasmodium* parasites, but these parasites can be gained during the blood meal on infected bird and can be an illustration of abortive haemosporidian development in not susceptible hosts [20].

The results obtained from blood of juvenile birds which hatched out in nest boxes on the Curonian Spit show that Paridae and Muscicapidae juvenile birds were infected with *Haemoproteus majoris* (hPARUS1, hCWT4, hPHSIB1, hWW2) and *Haemoproteus* sp. (hPARUS10) showing that the transmission of these parasite lineages takes place at the study site. We have found *H. majoris* (hCWT4) in *C. segnis* biting midges during this study. *Haemoproteus majoris* (hWW2) DNA was also recently found in *C. punctatus* in Lithuania [8], so biting midges of these two species can be considered as possible vectors of *H. majoris*. *Culicoides impunctatus* is known to be one of the natural vectors of *H. majoris* (hPARUS1)[10]. The prevalence of this genetic lineage was high in juveniles at the study site – up to 20.3 % of investigated *Cyanistes caeruleus* juveniles were infected with this parasite (Table 2). *Ficedula hypoleuca* juveniles were found not to be infected with *Haemoproteus* parasites showing that parasite transmission in this bird species may not take place at our study site.

Conclusion

The application of the new collection method revealed the species composition of ornithophilic *Culicoides* biting midges. Results obtained from the blood of juvenile birds have shown that transmission of *H. majoris* (hCWT4, hPARUS1, hPHSIB1, hWW2) and *Haemoproteus* sp. (hPARUS10) parasites take place at our study site. *Culicoides segnis*, *C. pictipennis*, and *C. kibunensis* being the dominant ornithophilic species and found to be infected with *Haemoproteus* parasites should be considered as possible vectors of these parasites.

The data on the possible *Haemoproteus* vectors can help to initiate detailed experimental studies of sporogony of various *Haemoproteus* spp. parasites with the most abundant ornithophilic biting midge species. Obtained information will be valuable for the knowledge on the vectors of haemosporidian parasites in Europe.

Declarations

Ethics approval

Experimental procedures of this study were approved by the International Research Cooperation Agreement between the Zoological Institute of the Russian Academy of Sciences and the Nature

Research Centre (1-12-2015–30-11-2020). All efforts were made to minimize handling time and potential suffering of animals.

Consent of publication

Not applicable.

Availability of data and materials

The data that support findings of this study are included within the article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

RZ, VP and RB – study conception and design; RZ, EP, AM, VP – fieldwork; RZ, EP – biting

midge dissection and microscopic examination; RB, EK, EP – molecular analysis; RZ, VP, RB – drafting of manuscript. All authors read and critically revised the manuscript and approved the final version.

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References

1. Wirth W. A Review of the Pathogens and Parasites of the Biting Midges (Diptera: Ceratopogonidae). *J Wash Acad Sci.* 1977; 67(2), 60–75.
2. Valkiūnas G. Avian malaria parasites and other haemosporidia. Boca Raton, USA: CRC Press; 2005.
3. Atkinson CT. *Haemoproteus*. In: Atkinson CT, Thomas NJ, Hunter BC, editors. Parasitic diseases of wild birds. Ames: Wiley-Blackwell; 2008. p. 13–35.
4. Donovan TA, Schrenzel M, Tucker TA, Pessier AP, Stalis IH. Hepatic hemorrhage, hemocoelom, and sudden death due to *Haemoproteus* infection in passerine birds: eleven cases. *J Vet Diagn Invest.*

2008;20(3): 304–13.

5. Ortiz-Catedral L, Brunton D, Stidworth MF, Elsheikha HM, Pennycott T, Schulze C, Braun M, Wink M, Gerlach H, Pendl, Gruber AD, Ewen J, Perez-Tris J, Valkiūnas G, Olias P. *Haemoproteus minutus* is highly virulent for Australasian and South American parrots. *Parasit Vector*. 2019;12:40.
6. Borkent A. World species of biting midges (Diptera: Ceratopogonidae). Accessed on: 2019-11-12.
7. Bukauskaitė D, Iezhova TA, Ilgūnas M, Valkiūnas G. High susceptibility of the laboratory-reared biting midges *Culicoides nubeculosus* to *Haemoproteus* infections, with review on *Culicoides* species that transmit avian haemoproteids. *Parasitology*. 2019;1–9.
8. Bernotienė R, Žiegytė R, Vaitkutė G, Valkiūnas G. Identification of a new vector species of avian haemoproteids, with a description of methodology for the determination of natural vectors of haemosporidian parasites. *Parasit Vector*. 2019;12:307.
9. Iezhova TA, Dodge M, Sehgal RN, Smith TB, Valkiūnas G. New avian *Haemoproteus* species (Haemosporida: Haemoproteidae) from African birds, with a critique of the use of host taxonomic information in hemoproteid classification. *J Parasit*. 2011; 97:682e694.
10. Žiegytė R, Markovets MY, Bernotienė R, Mukhin A, Iezhova TA, Valkiūnas G, Palinauskas V. The widespread biting midge *Culicoides impunctatus* (Ceratopogonidae) is susceptible to infection with numerous *Haemoproteus* (Haemoproteidae) species. *Parasit Vector*. 2017;10:397.
11. Carpenter S, Groschup MH, Garros C, Felipe-Bauer ML, Purse B. *Culicoides* biting midges, arboviruses and public health in Europe. *Antivir Res*. 2013;100:102–13.
12. Glukhova VM, Valkiūnas G. On the fauna and ecology of biting midges (Ceratopogonidae: Culicoides) in the Curonian spit, the methods of their collection from the birds and experimental infection with haemoproteids (Haemosporidia: Haemoproteidae). *Ekologija*. 1993;2:68–73.
13. Miltgen F, Landau I, Ratanaworabhan N, Yenbutra S. *Parahaemoproteus desseri* n. sp.; Gametogonie et shizogonie chez l'hôte naturel: *Psittacula roseate* de Thaïlande, et sporogonie expérimentale chez *Culicoides nubeculosus*. *Ann Parasitol Hum Comp*. 1981;56:123e130.
14. Ferraguti M, Martínez-de la Puente J, Ruiz S, Soriguer R, Figuerola J. On the study of the transmission network of blood parasites from SW Spain: diversity of avian haemosporidians in the biting midge *Culicoides circumscriptus* and wild birds. *Parasit Vector*. 2013; 6:208.
15. Bobeva A, Ilieva M, Dimitrov D, Zehindjiev P. 2014. Degree of associations among vectors of the genus *Culicoides* (Diptera: Ceratopogonidae) and host bird species with respect to haemosporidian parasites in NE Bulgaria. *Parasitol Res*. 2014;113(12):4505–11.
16. Synek P, Munclinger P, Albrecht T, Votýpka J. Avian haematophagous insects in the Czech Republic. *Parasitol Res*. 2013;112:839e845.
17. Santiago-Alarcón D, Havelka P, Pineda E, Segelbacher G, Schaefer HM. Urban forests as hubs for novel zoonosis: blood meal analysis, seasonal variation in *Culicoides* (Diptera: Ceratopogonidae) vectors, and avian haemosporidians. *Parasitology*. 2013;140(14):1799e1810.
18. Martínez-de la Puente J, Figuerola J, Soriguer R. Fur or feather? Feeding preferences of species of *Culicoides* biting midges in Europe. *Trends Parasitol*. 2015;31(1):16–22.

19. Bernotienė R. and Valkiūnas G. PCR detection of malaria parasites and related haemosporidians: the sensitive methodology in determining bird-biting insects. *Malar J.* 2016;15:283.
20. Valkiūnas G, Kazlauskienė R, Bernotienė R, Palinauskas V, Iezhova TA. Abortive long-lasting sporogony of two *Haemoproteus* species (Haemosporida, Haemoproteidae) in the mosquito *Ochlerotatus cantans*, with perspectives on haemosporidian vector research. *Parasitol Res.* 2013;112:2159–69.
21. Santiago-Alarcon D, Palinauskas V, Schaefer HM. Diptera vectors of avian haemosporidian parasites: untangling parasite life cycles and their taxonomy. *Biol Rev Camb Philos Soc.* 2012;87:928–64.
22. Tomas G, Merino S, Martinez-de la Puente J, Moreno J, Morales J, Lobato E. A simple trapping method to estimate abundances of blood-sucking flying insects in avian nests. *Anim Behav.* 2008;75:723e729.
23. Gutsevich AV. Blood-sucking midges of the genera *Culicoides* and *Forcipomyia* (Ceratopogonidae). *Fauna USSR.* 1st ed. Leningrad: Nauka Press; 1973.
24. Glukhova VM. Blood-sucking midges of the genera *Culicoides* and *Forcipomyia* (Ceratopogonidae). In: *Fauna of the USSR. Dipteran insects.* 1989;3(5a).
25. Mathieu B, Cêtre-Sossah C, Garros C, Chavernac D, Balenghien T, Carpenter S, Setier-Rio ML, Vignes-Lebbe R, Ung V, Candolfi E, Delécolle JC. Development and validation of ILC: an interactive identification key for *Culicoides* (Diptera: Ceratopogonidae) females from the Western Palaearctic region. *Parasit Vector.* 2012;5:137.
26. Dyce AL. The recognition of nulliparous and parous *Culicoides* (Diptera: Ceratopogonidae) without dissection. *Aust J Entomol.* 1969;8(1):11–5.
27. Žiegytė R, Palinauskas V, Bernotienė R, Iezhova TA, Valkiūnas G. *Haemoproteus minutus* and *Haemoproteus belopolskyi* (Haemoproteidae): complete sporogony in the biting midge *Culicoides impunctatus* (Ceratopogonidae), with implications on epidemiology of Haemoproteosis. *Exp Parasitol.* 2014;145:74–9.
28. Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol.* 1994;3:294–9.
29. Bensch S, Stjenman M, Hasselquist D, Ostman O, Hansson B, Westerdahl H, Pinheiro RT. Host specificity in avian blood parasites: a study of *Plasmodium* and *Haemoproteus* mitochondrial DNA amplified from birds. *Proc Royal Soc.* 2000;276:1583–9.
30. Hellgren O, Waldenstrom J, Bensch S. 2004. A new PCR assay for simultaneous studies of *Leucocytozoon*, *Plasmodium*, and *Haemoproteus* from avian blood. *J Parasitol.* 90:797–802.
31. Hellgren O, Bensch S, Malmqvist B. Bird hosts, blood parasites and their vectors-associations uncovered by molecular analyses of blackfly blood meals. *Mol Ecol.* 2008;17(6):1605–13.
32. Valkiūnas G, Iezhova TA, Križanauskienė A, Palinauskas V, Bensch S. A comparative analysis of microscopy and PCR-based detection methods for blood parasites. *J Parasitol.* 2008;94:1395–401.

33. Godfrey RD, Fedynich AM, Pence DB. Quantification of hematozoa in blood smears. *J Wildl Dis.* 1987;23:558–65.
34. Richardson DS, Jury FL, Blaakmeer K, Komdeur J, Burke T. Parentage assignment and extra group paternity in a cooperative breeder: the Seychelles warbler (*Acrocephalus sechellensis*). *Mol Ecol.* 2001;10:2263–73.
35. Hall TA. A user-friendly biological sequence alignment editor and analysis program for Windows 98/98/NT. *Nucleic Acid Symp Ser.* 1999;41:95–8.
36. Ciloglu A, Ellis VA, Bernotienė R, Valkiūnas G, Bensch S. A new one-step multiplex PCR assay for simultaneous detection and identification of avian haemosporidian parasites. *Parasitol Res.* 2019;118:191–201.
37. Pérez-Tris J, Bensch S. Diagnosing genetically diverse avian malarial infections using mixed-sequencing analysis and TA-cloning. *Parasitology.* 2005;131:15–23.
38. Carpenter S, Groschup MH, Garros C, Felipe-Bauer ML, Purse B. *Culicoides* biting midges, arboviruses and public health in Europe. *Antivir Res.* 2013;100:102–13.
39. Blackwell AA, Mordue J, Mordue W. Identification of bloodmeals of the Scottish biting midge, *Culicoides impunctatus*, by indirect enzyme-linked immunosorbent assay (ELISA). *Med Vet Entomol.* 1994;8:20–4.
40. Lassen SB, Nielsen SA, Kristensen M. Identity and diversity of blood meal hosts of biting midges (Diptera: Ceratopogonidae: *Culicoides* Latreille) in Denmark. *Parasit Vector.* 2012;5:143.
41. Lassen SB, Nielsen SA, Skovgård H, Kristensen M. Molecular identification of bloodmeals from biting midges (Diptera: Ceratopogonidae: *Culicoides* Latreille) in Denmark. *Parasitol Res.* 2011;108:823–9.
42. Ayllón T, Nijhof AM, Weiher W, Bauer B, Allène X, Clausen PH. Feeding behaviour of *Culicoides* spp. (Diptera: Ceratopogonidae) on cattle and sheep in northeast Germany. *Parasit Vectors.* 2014;7:34.
43. Trukhan MN, Tereshkina NV, Liutkevičius G. 2003. Peculiarities of the range of species and the ecology of midges (Diptera, Ceratopogonidae) on the Curonian spit. *Vesci nacyanalnaj akademii navuk Belarusi.* 2003;2:88–91.
44. Liutkevičius G. The new data on the epidemiology of bird haemoproteids (Haemosporida: Haemoproteidae) on the Curonian Spit. *Acta Zool Lithuan.* 2000;2:72–7.
45. Bobeva A, Zehindjiev P, Bensch S, Radrova J. A survey of biting midges of the genus *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) in NE Bulgaria, with respect to transmission of avian haemosporidians. *Acta Parasitol.* 2013. 58(4):585–91.
46. Palinauskas V, Iezhova TA, Križanauskienė A, Markovets MY, Bensch S, Valkiūnas, G. Molecular characterization and distribution of *Haemoproteus minutus* (Haemosporida, Haemoproteidae): a pathogenic avian parasite. *Parasitol Int.* 2013;62:358–63.
47. Olias P, Wegelin M, Zenker W, Freter S, Gruber AD, Klopffleisch R. Avian malaria deaths in parrots. *Eur Emerg Infect Dis.* 2011;17:950–2.

Tables

Table 1. Abundance of collected *Culicoides* biting midges and the prevalence of *Haemoproteus* parasites in biting midges obtained from nest boxes at the Neris Regional Park and at the Biological Station Rybachy

Species of biting midge	Neris Regional Park				Biological Station Rybachy			
	2017		2012		2018		2019	
	N*	P*(genetic lineage)	N*	P*(genetic lineage)	N*	P*(genetic lineage)	N*	P*(genetic lineage)
<i>C. festivipennis</i>	19						4	
<i>C. impunctatus</i>	1				1		5	
<i>C. kibunensis</i>	66	2 (pSW2, pSGS1)	11	1 (hRB1)	17		7	
<i>C. obsoletus gr.</i>	12		3				1	
			8		59	5	3	
<i>C. pictipennis</i>	7					(hTUPHI01)		
<i>C. reconditus</i>	1		14	1 (pSGS1)	2			
			24	2 (hSISKIN1, hTURDUS2)			2	
<i>C. segnis</i>	2	1 hCWT4						
<i>C. subfascipennis</i>	2							
<i>C. sphagnumensis</i>			2					
<i>C. punctatus</i>			1	1(hTURDUS2)				
<i>C. pallidicornis</i>			1					
<i>C. sp</i>	17						1	
Total:	127	3	64	5	79	5	23	0

N* - number of detected biting midges

P* - number of biting midges positive for haemosporidian parasites

Table 2. Prevalence of juvenile birds infected with *Haemoproteus* parasites, checked using microscopy and PCR-based methods.

Bird species	Analised/Infected	Parasite species, lineage	Prevalence (%)
<i>Cyanistes caeruleus</i>	74/18	<i>Haemoproteus majoris</i> , hPARUS1	15 (20.3)
		<i>H. majoris</i> , hCWT4	1 (1.4)
		<i>H. majoris</i> , hPHSIB1	2 (2.8)
<i>Parus major</i>	60/11	<i>H. majoris</i> , hPARUS1	5 (8.3)
		<i>H. majoris</i> , hPHSIB1	3 (5.1)
		<i>H. majoris</i> , hWW2	2 (3.4)
		<i>H. majoris</i> , hPARUS10	1 (1.7)
<i>Poecile palustris</i>	12/2	<i>H. majoris</i> , hPARUS1	1 (8.3)
		<i>H. majoris</i> , hPHSIB1	1 (8.3)
<i>Ficedula hypoleuca</i>	41/0	-	0 (0)

Figures



Figure 1

The collecting method of biting midges from bird nest boxes. Nest box attached to the tree (a); nestlings birds in the nest box (b); Petri dishe moisten with baby oil fixed upside down using double sided sticky tape on inside roofs of nest boxes (c); Petri dishe with sticking *Culicoides* spp. insects (d).