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Avian haemosporidian parasites (Haemosporida): sporogonic development and determination of vectors

DOCTORAL DISSERTATION

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GAMTOS TYRIMŲ CENTRAS

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Paukščių hemosporidiniai parazitai (Haemosporida): sporogoninis vystymasis ir pernešėjų nustatymas

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Contents

ABBREVIATIONS

dpi – days post infection
cytb – cytochrome *b* gene
DNA – Deoxyribonucleic acid
PCR – Polymerase Chain Reaction
RH – Relative Humidity
L/D – Light/Dark photoperiod

LIST OF PUBLICATIONS OF THE DISSERTATION TOPIC

This thesis is based on the following co-authored original publications with an impact factor on the Clarivate Analytics Web of Science database. Publications are referred to in the text by Roman numeral.

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II. Valkiūnas, G., Žiegytė, R., Palinauskas, V., Bernotienė, R., **Bukauskaitė, D.,** Ilgūnas, M., Dimitrov, D., Iezhova, T. 2015. Complete sporogony of *Plasmodium relictum* (lineage pGRW4) in mosquitoes *Culex pipiens pipiens*, with implications on avian malaria epidemiology. Parasitology Research 114, 3075–3085. (Q2, IF=2.558).

III. **Bukauskaitė D.,** Bernotienė R., Iezhova T.A., Valkiūnas G. 2016. Mechanisms of mortality in *Culicoides* biting midges due to *Haemoproteus* infection. Parasitology 143, 1748–1754. (Q2, IF=2.511).

IV. Valkiūnas G., Ilgūnas M., **Bukauskaitė D.,** Žiegytė R., Bernotienė R., Jusys V., Eigirdas, V., Fragner K., Weissenbock H., Iezhova T.A. 2016. *Plasmodium delichoni* n. sp.: description, molecular characterisation and remarks on the exoerythrocytic merogony, persistence, vectors and transmission. Parasitology Research 115, 2625–2636. (Q2, IF=2.558).

V. **Bukauskaitė, D.,** Iezhova, T.A., Ilgūnas, M., Valkiūnas, G. 2018. High susceptibility of the laboratory-reared biting midges *Culicoides nubeculosus* to *Haemoproteus* infections, with review on *Culicoides* species that transmit avian haemoproteids. Parasitology 1–9. DOI: https:// doi.org/10.1017/S0031182018001373. (Q2, IF=2.511).

AUTHOR CONTRIBUTIONS IN THE CORRESPONDING PAPERS

I. Experimental conception and design: GV (Gediminas Valkiūnas), **DB (Dovilė Bukauskaitė)**, RŽ (Rita Žiegytė); biting midge fieldwork: **DB**, RŽ, VP (Vaidas Palinauskas), DD (Dimitar Dimitrov), MI (Mikas Ilgūnas), RB (Rasa Bernotienė), GV; biting midge dissection and laboratory maintenance: **DB**, RŽ, RB; donor bird collection and testing: MYM (Mykhail Yu. Morkovets), TAI (Tatjana A. Iezhova), GV; phylogenetic analysis and its discussion VP, **DB**, GV; paper writing: **DB**, GV. All the authors read and approved the final version of the manuscript.

II. Experimental design: GV, VP; donor selection: VP, DD; maintenance of mosquito colonies: RŽ, RB, **DB**; experimental infections: **DB**, RŽ; laser microdissection: VP; molecular work, VP, MI, RB, **DB**; microscopic examination: RŽ, TI, **DB**; paper writing: GV.

III. Experimental conception and design: **DB**, GV; biting midges fieldwork: **DB**, GV, RB; donor bird selection: GV, TI; molecular work: RB, **DB**; paper writing: **DB**, GV.

IV. Donor bird collection and selection: GV, TI, VJ (Vytautas Jusys), VE (Vytautas Eigirdas); histological examination: MI, TI; Chromogenic in situ hybridisation: MI, KF (Karin Fragner), HW (Herbert Weissenbock); maintenance of mosquitoes colonies: RB, RŽ; experimental infections with mosquitoes: **DB**, RŽ; molecular work: RB, MI; paper writing: GV.

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LIST OF CONFERENCE PRESENTATIONS ON SUBJECT OF THE DISSERTATION

1. **Bukauskaitė, D.,** Valkiūnas, G., Žiegytė, R., Bernotienė, R., Palinauskas, V., Ježova, T. 2015. The influence of avian haemosporidian parasites on mosquito survival. *7th Europian Mosquito Control Associasion Workshop*. Valencia, Spain. Abstract book, 85 p. Oral presentation.

2. **Bukauskaitė, D.,** Žiegytė, R., Palinauskas, V., Iezhova, T., Dimitrov, D., Ilgūnas, M., Bernotienė, R., Markovets, YU. M., Valkiūnas, G. 2015. Biting midges (*Culicoides*, Diptera) transmit *Haemoproteus* parasites of owls: evidence from sporogony and molecular phylogeny. *6th Conference of the Scandinavian-Baltic Society for Parasitology "Current trends in parasitology*. Uppsala, Sweden. Oral presentation.

3. **Bukauskaitė, D.,** Bernotienė, R., Iezhova, T., Valkiūnas, G. 2016. Detrimental effects of *Haemoproteus* (Haemoproteidae) infection: ookinetes kill *Culicoides* biting midges. *EMOP XII-12th European Multicolloquium of Parasitology.* Turku, Finland. (http://congress.utu.fi/emop2016/). Oral presentation.

4. **Bukauskaitė, D.,** Žiegytė, R., Palinauskas, V., Dimitrov, D., Ilgūnas, M., Iezhova, T. A., Bernotienė, R., Markovets, M. Yu., Valkiūnas, G. 2016. *Culicoides nubeculosus* is an effective vector of avian haemoproteids. *3 rd international conference "Malaria and related haemosporidian parasites of wildlife.* Arbanasi, Bulgaria. Abstract book, 9 p. Oral presentation.

5. **Bukauskaitė, D.,** Iezhova T. A., Ilgūnas M., Valkiūnas G. 2017. Development of *Haemoproteus* species in the laboratory reared biting midge *Culicoides nubeculosus*. *7th Conference of the Scandinavian-Baltic Society for Parasitology.* Riga, Latvia. Abstract book, 16 p. (http://csbsp7.mozello.com/). Oral presentation.

AWARDS

1. Research Council of Lithuania. Scholarship for academic achievement (2014, 2015, 2016 and 2017 years).

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SCIENTIFIC PROBLEM

Haemosporidian parasites belonging to genera *Haemoproteus* and *Plasmodium* (Haemosporida, Haemoproteidae) are important pathogens of birds due to their high prevalence in many bird populations, diseases and even mortality caused in some avian hosts (Garvin et al., 2003; Donovan et al., 2008; Olias et al., 2011; Pacheco et al., 2011; Palinauskas et al., 2015). Much information is available about the prevalence (Silva-Iturriza et al., 2012; Ishtiaq et al., 2007; Latta and Ricklefs, 2010), genetic diversity (Dimitrov et al., 2010; Belo et al., 2011; Szymanski and Lovette, 2005; Ivanova et al., 2015) and phylogenetic relationships (Santiago-Alarcon et al., 2010; Carlson et al., 2013; Yoshimura et al., 2014; Bensch et al., 2016) of avian haemoproteids, but vectors, sporogonic development and patterns of transmission of these blood parasites remain insufficiently investigated. This is a prominent obstacle for a better understanding of the epidemiology of diseases caused by these pathogens and the development of preventive measures (**Paper V**).

Haemoproteus parasites are transmitted by louse flies (Hippoboscidae) and biting midges (Ceratopogonidae). Species of subgenus *Parahaemoproteus* are transmitted by *Culicoides* biting midges (Garnham, 1966; Valkiūnas, 2005; Atkinson et al., 2008). Recent experimental studies have showed that the widespread biting midge *Culicoides impunctatus* is susceptible to many *Parahaemoproteus* species (Žiegytė et al., 2017), but information about the vectorial capacity of other biting midges remains scanty mainly because experimental work with these insects is complicated due to a short period (several weeks in spring-summer time) when they are abundant in the wild in Europe (Liutkevičius, 2000). High mortality of wild-caught biting midges in captivity also complicates experimental research with these insects (Valkiūnas and Iezhova, 2004). That limits opportunities of experimental research in the wild and calls for laboratory research using colonies of biting midges. Experimental observations on sporogony in laboratory-reared biting midges would be helpful and would speed up vector studies, but the methodology of such research remains insufficiently developed. *Culicoides nubeculosus* is widespread in Europe (Mathieu et al., 2012). This insect was colonized (Boorman, 1974) and is susceptible to several *Haemoproteus* species (Miltgen et al., 1981; Žiegytė et al., 2016). However, the range of *Haemoproteus* parasites which can complete sporogony in *C. nubeculosus* remains unclear (**Papers I and V**).

It is important to note that the detection of sporozoites in insects remains crucial for the final identification of vectors, because PCR-based

methods could detect abortive sporogonic development (Valkiūnas et al., 2013) (**Papers I and V**).

Many studies have reported the reduced survival of wild birds infected with *Haemoproteus* parasites and the negative effects of this infection on immunity indices, body condition, and reproductive success of hosts (Marzal et al., 2005; Valkiūnas et al., 2006; La Puente et al., 2010). However, little is known about the effects of *Haemoproteus* infections on blood-sucking insects. Experimental studies showed that high parasitaemia of *Haemoproteus* species is lethal to *Culicoides* midges. Liutkevičius (2000) and Valkiūnas and Iezhova (2004) reported high mortality of *C. impunctatus* infected with *Haemoproteus balmorali*, *H. belopolskyi*, *H. dolniki, H. fringillae, H. lanii* and *H. tartakovskyi*. However, mechanisms of mortality remain unclear. It was speculated (Valkiūnas and Iezhova, 2004) that the mortality of biting midges might be due to the damage caused by ookinetes and/or developing oocysts, which injure the epithelial cells of the midgut and might cause associated inflammatory reactions. However, histological observations are absent in biting midges (**Paper III**).

Species of *Plasmodium* cause malaria in birds (Garnham, 1966; Valkiūnas et al., 2008; Mantilla et al., 2013; Ilgūnas et al., 2013; Walther et al., 2014). In all, 55 species of *Plasmodium* infecting birds have been recognized (Valkiūnas and Iezhova, 2018). However, vectors of these parasites of species levels remain insufficiently investigated.

Plasmodium relictum (Haemosporida, Plasmodiidae) causes malaria in birds and is the first in the frequency of occurrence of avian malaria parasites reported from over 300 species of birds at all continents, except the Antarctic (Garnham, 1966; Valkiūnas, 2005; Atkinson et al., 2008; Valkiūnas et al., 2018). Two *P. relictum* lineages (pSGS1 and pGRW4), which are widely distributed both by hosts and geographically (Beadell et al., 2006; Ejiri et al., 2009; Clark et al., 2014; Perkins, 2014), have been reported in birds all over the world, but areas of their transmission are different (Marzal et al., 2011). The lineage pSGS1 is cosmopolitan in transmission (Bensch et al., 2009; Marzal et al., 2015). *Culex pipiens* mosquitoes are effective vectors of this parasite (Vézilier et al., 2010; Kazlauskienė et al., 2013), and sporogony completes at a temperature of 12-30 °C (Garnham, 1966; Valkiūnas, 2005; Žiegytė et al., 2014a). As to the lineage pGRW4, it has been reported to be actively transmitted mainly in countries with warm climates (Ricklefs et al., 2004; Beadell et al., 2006; Bensch et al., 2009; Marzal et al., 2011; Loiseau et al., 2012). *Culex quinquefasciatus* is an effective vector of this parasite (Atkinson et al., 2008; LaPointe et al., 2010; Freed and Cann, 2013), but this insect is absent from

temperate regions of Europe where *C. pipiens* mosquitoes act as active vectors of avian malaria (Vézilier et al., 2010; Kazlauskienė et al., 2013; Cornet et al., 2013). It remains unclear if the lineage pGRW4 can complete sporogony in *C. pipiens* form *molestus* and other widespread European mosquitoes and if that happens at relatively low temperatures. We address this issue in this study (**Paper II**).

A new malaria parasite *Plasmodium* (*Novyella*) *delichoni* (Haemosporida, Plasmodiidae) was recently discovered (Valkiūnas et al., 2016) in a widespread Eurasian songbird, the Common House Martin *Delichon urbicum* (Hirundinidae). Because vectors of this infection are unknown, experiments are needed to identify its potential vectors. (**Paper IV**).

OBJECTIVES AND MAIN TASKS OF THE STUDY

The objectives of this study were to examine the sporogonic development of widespread *Haemoproteus* and *Plasmodium* parasites in wild-caught and laboratory-reared blood-sucking dipteran insects and to determine vectors of these infections.

The following tasks were set to achieve these objectives:

1. To develop a method for experimental infections of laboratory-reared biting midges *Culicoides nubeculosus* with avian *Haemoproteus* parasites.
2. To investigate sporogonic development and determine experimental vectors of *Haemoproteus* parasites of owls.
3. To investigate sporogonic development and determine vectors of three widespread *Haemoproteus* parasites of passerine birds.
4. To determine phylogenetic relationships among *Haemoproteus* species, which vectors have been identified.
5. To investigate the mechanism of mortality of *Culicoides impunctatus* biting midges due to *Haemoproteus lanii* infection.
6. To determine if the sporogonic development of *Plasmodium relictum* (lineage pGRW4) and *Plasmodium delichoni* completes in laboratory-reared mosquitoes.

STATEMENTS TO BE DEFENDED

1. The new method is appropriate to be used for experimental infections of laboratory-reared biting midges *Culicoides nubeculosus* with avian *Haemoproteus* parasites.
2. Two parasites of owls (*Haemoproteus noctuae* and *H. syrnii*) complete sporogony in laboratory-reared biting midges *Culicoides nubeculosus* and wild-caught *C. impunctatus*, and these insects are likely natural vectors.
3. The sporogony of three widespread *Haemoproteus* parasites of passeriform birds completes in experimentally infected biting midges *Culicoides nubeculosus*, and this insect is likely a natural vector.
4. Phylogenies based on cytochrome *b (cytb)* gene indicate possible vectors (biting midges or louse flies) of avian haemoproteid parasites.
5. Migrating ookinetes, but not oocysts, are the main cause of death in biting midges infected with high *Haemoproteus lanii* gametocytaemia.
6. The sporogonic development of *Plasmodium relictum* (lineage pGRW4) completes in laboratory-reared mosquitoes *Culex pipiens* form *molestus*, but *Plasmodium delichoni* does not develop in *Culex quinquefasciatus*, *Culex pipiens* form *molestus,* and *Aedes aegypti*.

NOVELTY OF THE STUDY

1. An easy to use method was developed for the experimental infections of laboratory-reared biting midges *Culicoides nubeculosus*. Using this method it was determined that 5 *Haemoproteus* species (*H. noctuae*, *H. syrnii*, *H. minutus*, *H. motacillae,* and *H. attenuatus*) complete sporogonic development in this insect. Furthermore, it was determined that phylogenies based on *cytb* gene readily indicate the possible vectors of avian haemoproteids.
2. It was determined that high *Haemoproteus* infections are lethal to biting midges due to damage caused by migrating ookinetes in abdomen and thorax.
3. This study showed for the first time that *Plasmodium relictum* (lineage pGRW4) completes sporogony in mosquitoes *Culex pipiens* form *molestus* and *Culex quinquefasciatus* with similar patterns of

development, and *Plasmodium delichoni* development is abortive in mosquitoes *Culex pipiens* form *molestus*, *Culex quinquefasciatus,* and *Aedes aegypti*.

1. BRIEF LITERATURE REVIEW

Malaria is an ancient disease. The early Greeks noticed that people who lived near marshy areas had poor health, malarial fevers and enlarged spleens. Later on, in 1676, Antoni van Leeuwenhoek discovered bacteria-like organisms, and in 1878-1879 Louis Pasteur and Robert Koch determined that these microorganisms cause infectious diseases. Due to these discoveries, the search for the causative agents of malaria has intensified. In 1897, Ronald Ross identified malaria vectors. Working in India, he discovered that Culicinae mosquitoes transmitted the avian malaria parasite *Plasmodium relictum,* and he described the life cycle of *P. relictum* in vectors (Cox, 2010). After this discovery, domestic canaries started to be used in avian haemosporidian parasite research.

For more than 100 years, haemosporidian parasites have been studied mainly using microscopic methods. However, it is not always possible to identify the parasite species by using blood slides even by experts, because the intensity of parasitemia is often low or not all stages which are necessary for identification are present in blood films (Valkiūnas, 2005). When molecular techniques began to be used in this field of research, a huge leap in haemosporidian parasite diversity was achieved. In 2002, the genome of *Plasmodium falciparum* was sequenced (Gardner et al., 2002). The development of an open access database (http://mbio-serv2.mbioekol.lu.se/Malavi/) for avian haemosporidian parasite sequences (MalAvi) was established (Bensch et al., 2009), and this provided new opportunities for avian haemosporidian biodiversity, ecology and evolutionary biology studies.

The life cycle of haemosporidian parasites is characterized by a set of complex and strictly ordered stages occurring in vertebrate hosts and vectors. The development takes place in two hosts: the vertebrates that are intermediate hosts and the vectors (blood-sucking insects) that are final hosts. After feeding on infected birds, the gametocytes get into the midgut of the vector and escape from the host cells. Microgametes are formed and fertilization occurs extracellularly. After fertilization, a zygote is formed and develops into an elongated ookinete, which penetrates through peritrophic membrane and through the epithelial layer of the midgut. The ookinete rounds up under the basal lamina and develops into an oocyst. Numerous uninuclear elongated bodies (sporozoites) are formed in the oocyst. After maturation of oocysts, the sporozoites move into the haemocoel and then penetrate the salivary glands of the vector. Sporozoites are infective to birds. Transmission occurs when sporozoites are injected by the vector into

vertebrate hosts with salivary gland secretion during its blood meal (Valkiūnas, 2005; Atkinson et al., 2008).

Vectors of *Haemoproteus* parasites are biting midges. *Culicoides nubeculosus* is widely distributed in the Palearctic (Mathieu et al., 2012) and probably is a natural vector of avian haemoproteids. This species was colonized (Boorman, 1974) and is susceptible to several *Haemoproteus* species (Miltgen et al., 1981; Žiegytė et al., 2016). According to limited available data, *C. nubeculosus* is not specialized in blood meal. This biting midge willingly takes blood from various mammals and birds (Jennings and Mellor, 1988). Recent experimental studies, which applied experimental exposure of sheep (Pages et al., 2014), canaries (Svobodova et al., 2017) and various species of wild passeriform birds (Žiegytė et al., 2016), support this conclusion. Further field studies using molecular markers are needed for a better understanding of feeding preferences of *C. nubeculosus* in different ecosystems. *Culicoides impunctatus* is widespread in the Palearctic and abundant in Europe (Glukhova and Valkiūnas, 1993; Blackwell et al., 1999; Patakakis et al., 2009). *Culicoides impunctatus* was often considered to be mainly a mammalophilic species (Blackwell et al., 1994), but it willingly takes blood meal on birds (Žiegytė et al., 2014b; Bernotienė et al., 2016; Glukhova and Valkiūnas, 1993; Liutkevičius, 2000) and is a vector of 11 *Haemoproteus* parasites (Žiegytė et al., 2017).

Malaria vectors are mosquitoes (Insecta: Diptera) belonging to the family Culicidae. *Culex* females are vectors of many species of avian malaria parasites (Valkiūnas, 2005). *Culex pipiens* mosquitoes, which are distributed worldwide, are effective vectors of pathogenic *P. relictum* parasites, which are of cosmopolitan distribution (Vézilier et al., 2010; Kazlauskienė et al., 2013).

Modern polymerase chain reaction (PCR-based) molecular methods are broadly used in haemosporidian parasite research. The genetic diversity of parasites is relatively well studied (Bensch et al., 2009). Numerous new cytochrome *b* lineages of avian haemosporidians have been determined (Bensch et al., 2000; 2009). Partial sequences of other genes also were studied, particularly an apicoplast gene (ClpC) (Santiago-Alarcon et al., 2010) and a nuclear DHFR-TS gene (Bensch et al., 2004). Phylogenetic relationships of parasites have been addressed in many studies (Santiago-Alarcon et al., 2010; Carlson et al., 2013; Bensch et al., 2016; Olsson-Pons et al., 2015; Yoshimura et al., 2014), but are insufficiently investigated in regard of vectors, which remain unidentified for the majority of described wildlife haemosporidian parasite species. Because knowledge about avian haemosporidian parasite vectors is insufficient, we have designed this study

aiming to create new knowledge about sporogonic development and determination of vectorial capacity of blood-sucking dipteran insects that are common in Europe and abundant in Lithuania.

2. MATERIAL AND METHODS

A detail description of material collected and methods used in this dissertation is given in published papers (**Papers I-V**). Below, a brief description of this information is given.

2.1. Study sites

The fieldwork and experimental infections of blood-sucking insects with *Haemoproteus* parasites were carried out at Ventės Ragas Ornithological Station, Lithuania (https://vros.lt) in May of 2015 and 2017 (**Paper V**) and at the Biological Station of the Zoological Institute of the Russian Academy of Sciences on the Curonian Spit in the Baltic Sea (55°05' N, 20°44' E) between 9th and 24th June in 2014 (**Paper I** and **III**).

Experimental infections with *Plasmodium* parasites were carried out at P. B. Šivickis Laboratory of Parasitology, Nature Research Centre (**Paper II** and **IV**). All material collected during PhD studies was examined and analyzed at Nature Research Centre, Vilnius.

2.2. Selection of parasite donors for vector research

During fieldworks, birds were caught with mist nets, "Zigzag" traps and a big "Rybachy"-type trap. Birds were ringed, and approximately 30 µl of blood was collected with microcapillaries by punching the brachial vein. One drop was used to make three blood films. The smears were air-dried within 5-15 seconds after their preparation, fixed in absolute methanol and stained with a 10% Giemsa solution, as described by Valkiūnas (2005). Remaining blood was stored in a non-lysis SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0) for molecular analysis.

The blood smears were examined microscopically to identify parasite species and to control for possible presence of natural co-infections with other haemosporidian parasites. Preparations were examined with an Olympus BX-43 light microscope equipped with an Olympus SZX2-FOF digital camera and imaging software QCapture Pro 6.0, Image-Pro plius (Tokyo, Japan). Blood smears were examined for 15-20 min at low

magnification (x 400), and approximately 100 fields were studied at high magnification (x 1000). Intensity of gametocytaemia was determined as a percentage by actual counting of the number of mature gametocytes per 1000 red blood cells. All vector preparations were examined at high (x 1000) magnification. Parasite species were identified according to Valkiūnas (2005) (**Papers I-V**).

For *Plasmodium* vector research, experimental birds were infected with blood collected from naturally infected birds. These birds were exposed by subinoculation of about 250 µl of freshly prepared mixture containing infected blood, 3.7% sodium citrate (anticoagulant) and 0.9% saline (4:1:5) into their pectoral muscle. Before subinoculation experiments, all recipient birds had been proven to be uninfected with haemosporidian parasites by microscopic examination of blood films and later by PCR-based testing in the laboratory (**Papers II** and **IV**).

2.3. Dipteran insects used in experimental research

2.3.1. Colony of *Culicoides nubeculosus*

To determine the sporogonic development and possible vectors of *Haemoproteus* species, *Culicoides nubeculosus* colonies were used. Biting midges were reared in the laboratory at the Nature Research Centre according to Boorman (1974). Each insect colony was maintained in small (approximately 5 cm in diameter) cardboard boxes covered by fine mesh bolting silk. Each box contained approximately 50-70 individuals of biting midges. The laboratory-reared biting midges were delivered to the field (**Paper I** and **V**) for experimental infections.

2.3.2. Wild-caught *Culicoides impunctatus* biting midges

To examine the effects of *Haemoproteus* infection on survival and to investigate owl parasite vectors, wild *Culicoides impunctatus* were used (**Paper I** and **III**). Experimental infection was done near Lake Chaika, located close to the Rybachy village (Kaliningrad, Russia) between 9th and 24th June. According to Liutkevičius (2000), six species of biting midges were found in the study area (*Culicoides reconditus*, *C. obsoletus*, *C. pictipennis*, *C. punctatus*, *C. segnis,* and *C. impunctatus*), but the most abundant was *C. impunctatus*, 94.6% of the total number of collected specimens. The latter insect was used in our experiments.

2.3.3. Colonies of mosquitoes

For investigation of the sporogonic development of *Plasmodium* parasites, laboratory-reared mosquito colonies were used (**Paper II** and **IV**). *Culex pipiens* form *molestus* were obtained from Dr. Roland Kuhn. The colony was originally started from larvae collected in Hesse Region (Germany). Colonies of *C. quinquefasciatus* and *Aedes aegypti* mosquitoes were established using eggs provided by Dr. Ana Rivero (France) and Dr. Hilary Ranson (UK), respectively. The insects were colonized as described by Žiegytė et al. (2014a). Briefly, mosquitoes were kept in cages (120x45x45 cm) under standard conditions (65-70% RH and L/D photoperiod of 16/8 h). Several pads of cotton wool moistened in a 10% sugar solution and a water bowl for oviposition were placed in each cage for *C. pipiens* form *molestus* and *C. quinquefasciatus* colonies. For *A. aegypti,* a bowl with wet cotton covered with filter paper was placed in each box for laying eggs.

2.4. Experimental design

2.4.1. Experimental infection of *Culicoides nubeculosus* biting midges with *Haemoproteus* parasites

Culicoides nubeculosus biting midges were reared in the laboratory according to Boorman, (1974). Briefly, they were kept in small cardboard boxes covered with fine mesh bolting silk. Each box contained approximately 50-70 individuals of biting midges. Birds naturally infected with single *Haemoproteus* infections were used as donors of gametocytes to expose biting midges. A box with unfed *C. nubeculosus* was gently pressed to the feather-free area on pectoral muscles of infected birds (Fig. 1 in **Paper I**). The midges willingly took blood meal through the bolting silk, and majority of them were fully engorged after approximately 30-40 min. Then, the experimental insects were released into a cage made of bolting silk (12x12x12 cm), and males and non-fed females were removed. The remaining engorged biting midges were kept in a room with controlled temperature (22 °C), humidity (75±5%) and L/D photoperiod (17:7h). Cotton pads moistened with 10% solution of sugar were placed on the top of each cage in order to feed insects daily (**Paper I** and **V**).

2.4.2. Experimental infection of wild *Culicoides impunctatus* biting midges with *Haemoproteus* parasites

Wild-caught biting midges *C. impunctatus* were exposed by allowing them to take blood meals on selected birds as described by Valkiūnas (2005). Briefly, the feathers from a surface of about 1 cm^2 of the birds' heads were gently plucked off. Birds were kept in hands covered with rubber gloves at a site with a high density of biting midges, which were allowed to feed naturally on the feather-free area. The birds were exposed to bites of biting midges between 10 and 12 pm. When several females began taking blood meals on a bird head, the head with feeding insects was carefully placed into an unzipped insect cage (12x12x12 cm) made of fine mesh bolting silk. The engorged females flew off after feeding. The cage with engorged biting midges was then closed using a zipper. Cages with engorged insects were placed in plastic packs and transported to the laboratory. Pads of cotton wool moistened with a 10% saccharose solution were placed on the top of each insect cage. Both infected and control groups of biting midges were held in standard conditions (16-18 °C, 70±5% RH and L/D photoperiod of 17:7 h) (**Paper I** and **III**).

2.4.3. Experimental infection of mosquitoes with *Plasmodium* parasites

Two days before infection, approximately 30 unfed females of each species were randomly chosen and placed inside separate experimental cages of 45x45x45 cm. To increase favour of blood feeding, the experimental mosquitoes were deprived of sugar for 24 hours. Experimental birds with gametocytaemia ranging between 0.1 and 2% were placed in mosquito cages and exposed, as described by Kazlauskienė et al. (2013). Briefly, infected birds were placed in plastic tubes (length 15 cm, diameter 5 cm) containing a rip, which was used to fix bird legs. Both tube ends were covered with bolting silk. Only legs were exposed to mosquito bites. The birds were kept in insect cages for approximately 15-20 min every 3-4 days. All mosquito species willingly took blood meals. We evaluated parasitemia in all donor birds immediately after mosquito blood meals. Engorged females were taken from the experimental cages by using an aspirator, placed in separate small insect cages (12x12x12 cm), maintained at the same conditions as their colonies to allow the development of parasites, and dissected in intervals (**Paper II** and **IV**).

2.5. Dissection of insects and making preparations of sporogonic stages

Experimentally infected insects were dissected at intervals, and preparations of ookinetes (**Papers I-V**), oocysts and sporozoites (**Papers I, II, IV** and **V**) were prepared. Engorged females were anesthetized by putting them into a tube with a cotton pad wetted in 96% ethanol.

Parasite preparations were prepared according to Valkiūnas (2005). Briefly, the midguts and salivary glands were extracted, gently crashed to prepare small smears, air dried, fixed with methanol, and stained with Giemsa. Midgut preparations were stained in the same way as blood smears, while 4% staining solution was used to stain preparations of salivary glands. Oocysts were first visualized by adding a minute drop of 2% mercurochrome solution on a freshly prepared midgut preparation, which was then covered with a coverslip. That simplified the search of tiny *Parahaemoproteus* parasite oocysts. Oocyst-infected midguts were fixed in 10% formalin solution for 24 h; formalin was then replaced with 70% ethanol. After 6 h, the midgut preparations were washed with distilled water, stained with Ehrlich's hematoxylin for 10 min, steeped in water containing a pinch of sodium bicarbonate and differentiated with acid ethanol both for 5 min, and again steeped in water with sodium bicarbonate. Then, each preparation was dehydratated with 70% and 96% ethanol, cleared by putting a drop of clove oil and xylene over the preparation, and finally mounted in Canada balsam and covered with a cover slip.

2.6. Molecular methods

2.6.1. Polymerase chain reaction and sequencing

Total DNA was extracted from all samples by using the ammonium acetate extraction method (Richardson et al., 2001). For genetic analysis, we used a nested PCR protocol (Bensch et al., 2000; Hellgren et al., 2004). Amplification of the *cytb* gene was done using two pairs of primers: HaemNFI and HaemNR3 for detection of *Haemoproteus*, *Plasmodium* and *Leucocytozoon* species; and 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 positive by microscopic examination of blood films) were used per every 14 samples. No cases of false positive or negative samples were reported. To support species identification of wild-caught biting midges, we

used the insect-specific primers LCO149 and HCO2198, which amplify a fragment of cytochrome oxidase subunit I of mitochondrial DNA (Folmer et al. 1994). Fragments of DNA from the PCR positive samples were sequenced from the 3′ and 5′ ends. The genetic analyser "Basic Local Alignment Search Tool" (National Centre of Biotechnology Information website: http//www.ncbi.nlm.nih.gov/BLAST) was used to determine the lineages of detected DNA sequences. Sequences were edited and aligned using BioEdit (Hall, 1999) and deposited in GenBank (**Papers I-V**).

2.6.2. Phylogenetic analysis

To determine phylogenetic relationships among investigated *Haemoproteus* parasites and other avian haemoproteids for which vectors have been identified, we constructed a phylogenetic tree using sequences of the mitochondrial *cytb* gene of *Haemoproteus* spp. and *Plasmodium* spp.; each sequence was of 479 bp. The tree was created using Bayesian phylogenetics as implemented in mrBayes version 3.1 (Ronquist and Heulsenbeck, 2003). The best-fit model of evolution (GTR + I + G) was selected by software Modeltest 3.7 (Posada and Crandall, 1998). We ran two independent analyses with a sample frequency of every 100th generation over 12 million generations. For the construction of the majority consensus tree, 25% of the initial trees in each run were discarded as burn-in periods. We visualized the tree by using the software Tree View 1.6.6. (available from <http://evolution.genetics.washington.edu/phylip/software.html>). The sequence divergence between the different lineages was calculated with the use of a Jukes-Cantor model of substitution, with all substitutions weighted equally, implemented in Molecular Evolutionary Genetics Analysis (MEGA) software, version 4.0 (Tamura et al., 2007) (**Papers I** and **V**).

2.7. Statistical analyses

The statistical analyses were carried out using the "Statistica 7" package (**Paper I-III**) and "R studio" version 3.4.3 (**Paper V**). Student's t-test for independent samples was used to determine statistical significance between mean linear parameters of parasites. Percentages of survived control and experimental insects were compared by Fisher's exact test. In all comparisons, a P value of 0.05 or less was considered significant.

2.8. Ethical statement

Experimental procedures 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 the Institute of Ecology of Nature Research Centre (25.05.2010) (**Papers I-IV**) and by the Ethical Commission of the Baltic Laboratory Animal Science Association, Lithuania; Lithuanian State Food and Veterinary Office (permit no. G2-27, 07.05.2015); and Environmental Protection Agency, Vilnius (permits no. 21, 08.04.2015 and no. 25, 27.04.2015 (**Paper IV**) and permits no 21, 05.04.2015 and no. 23, 26.04.2017 (**Paper V**)). The Kaliningrad Zoo provided one naturally infected tawny owl for experimental research according to the ethical approval of 05.06.2014 (**Paper I**).

All efforts were made to minimize handling time and potential suffering of birds. None of the experimental birds suffered apparent injury during experiments, and wild birds were released after experiments.

3. RESULTS AND DISCUSSION

3.1. Development of a method for experimental infections of colonized *Culicoides nubeculosus* biting midges with avian *Haemoproteus* parasites

Miltgen et al. (1981) experimentally exposed *Culicoides nubeculosus* to *Haemoproteus handai* (syn. *H. desseri*) parasite and demonstrated the development of numerous sporozoites for the first time. However, the experimental design is not described in this paper. We have developed a method which is described in the Material and Method Section briefly. We described this method in detail in **Papers I** and **V**. The new method developed is easy to use in experimental infections with *Haemoproteus* parasites.

3.2. Sporogonic development of *Haemoproteus* parasites of owls in biting midges

We determined and described in **Paper I** that two parasites of owls completed the sporogonic development in experimentally infected biting midges. Ookinetes, oocysts and sporozoites of *Haemproteus noctuae* were seen both in experimentally infected wild-caught *Culicoides impunctatus* and laboratory-reared *C. nubeculosus* biting midges, indicating completed sporogony. *Haemoproteus syrnii* completed sporogony in *C. nubeculosus*. Morphologically identical sporozoites of *H. noctuae* developed both *C. impunctatus* and *C. nubeculosus*. There was no significant difference in the length, width, area or area of nuclei in *H. noctuae* sporozoites, which developed into different species of biting midges ($P > 0.05$ for all these features). Additionally, sporozoites of *H. syrnii* were significantly shorter and smaller in area than those of *H. noctuae* ($P < 0.05$ for both these features).

Both these parasites completed sporogony in *Culicoides* biting midges, with sporozoites reported in salivary glands, indicating that these flies are likely natural vectors, as has been reported for *Parahaemoproteus* species (Atkinson et al., 1988; Valkiūnas, 2005; Žiegytė et al., 2014b). The demonstration of sporozoites in salivary glands is an essential step for definitive demonstration that insects can act as vectors (Žiegytė at al., 2014b; Valkiūnas et al., 2002; Atkinson, 1991; Fallis and Wood, 1957).

3.3. Sporogonic development of three widespread *Haemoproteus* parasites of passerine birds in laboratory-reared *Culicoides nubeculosus* biting midges

We determined and described in **Paper V** that the sporogony of all parasite species used in the experiment occurred and completed in experimentally infected biting midges *Culicoides nubeculosus*. The sporozoites of *Haemoproteus minutus*, *H. motacillae* and *H. attenuatus* developed and were visualized in a salivary glands preparation (see Fig 1 m-o, in **Paper V**). Based on morphological characteristics, sporozoites can be readily distinguished from each other in *C. nubeculosus* biting midges.

In all, the available data shows that seven species of avian haemoproteids parasitizing birds of three orders can use *C. nubeculosus* as the final host and as a vector. In other words, this insect is highly susceptible to various *Haemoproteus* infections, and it most likely participates in the natural transmission of haemoproteosis. Wild-caught *C. impunctatus* insects were used in many experimental studies (Žiegytė et al., 2014b; Žiegytė et al., 2017). However, experimental research with wild-caught *C. impunctatus* insects is limited due to a short period (several weeks in spring-summer time) when they are very abundant and are easy to sample in large numbers for experimental infections at many study sites in Europe (Liutkevičius, 2000). This complicates the use of wild-caught insects in experimental research. Additionally, the mortality of wild-caught *C. impunctatus* insects is high in captivity (Valkiūnas and Iezhova, 2004). Meanwhile, *C. nubeculosus* is easy to rear in laboratory conditions and survives well (Boorman, 1974). We recommend using this insect in experimental *Haemoproteus* parasite studies, which can be designed and carried out all year round. This opens new opportunities for delicate experimental observations on various aspects of haemoproteid parasites biology.

3.4. Analysis of phylogenetic relationships of *Culicoides* species transmitting avian *Haemoproteus* parasites

All species of *Haemoproteus* used in this study (**Paper I** and **V**) appeared in one well-supported clade with other *Culicoides* species-transmitted parasites. These parasites belong to subgenus *Parahaemoproteus*. The species of subgenus *Haemoproteus,* which are transmitted by louse flies, appeared in a separate well-supported clade (Fig. 2 in **Paper V**).

Based on obtained results, we predict that all *cytb* sequences of haemoproteids transmitted by biting midges will be grouped with the closely related lineages of *Culicoides* spp.-transmitted parasites in one clade, and the lineages of louse flies-transmitted parasites will be clustered with corresponding lineages in another clade. In other words, the phylogenetic analyses of *cytb* sequences provide opportunities to predict groups of vectors (species of Ceratopogonidae or Hippoboscidae) which are involved in the transmission of avian haemoproteids.

A relatively strict linkage of haemosporidian parasites of particular clades to specific insect families remains insufficiently understood, and there is no convincing explanation of this observation. It might be an indication that development of vectors may be essential in the selection of the drive of evolution in mtDNA genes in haemosporidian parasites. These protists use both glycolysis and oxidative phosphorylation energy metabolism during their life cycle. However, glycolysis predominates during haemosporidian development in the vertebrate host, but the parasites switch mainly to oxidative phosphorylation in the insect vectors, in which glucose is insufficiently available for adenosine triphosphate synthesis (Hino et al., 2012). Thus, the mitochondrial genes are crucial for the survival of haemosporidians in vectors, but not so important during development in vertebrates, in which glycolysis predominates (Hall et al., 2005; Jacot et al., 2016; Pacheco et al., 2018). The phylogenies based on mtDNA may reflect well the parasite-vector evolutionary relationships, but not so well the models of evolution of the entire 'vertebrate host-parasite-vector' system. That might explain the contradictions in haemosporidian phylogenies based on different genes (Bensch et al., 2016). Analysis of phylogenies based on complete haemosporidian genomes is needed to answer this question.

3.5. Mechanisms of mortality of *Culicoides impunctatus* biting midges due to *Haemoproteus* infection

In **Paper III** we determined the survivorship and mechanisms of mortality of wild *Culicoides impunctatus* infected with *Haemoproteus lanii* (hRB1) with high gametocytaemia (5.2%). The survivorship of control and infected groups differed significantly (Fisher's exact test, $P < 0.001$). Most of infected biting midges were dead (98%) within 12 hours after infection. Mature ookinetes were numerous in preparations of midgut contents and in haemocoel in the histological section of abdomen and thorax (see Fig. 1 in **Paper III**).

Mature ookinetes of different *Haemoproteus* species develop within 2-12 h after infection to air both "*in vitro*" and "*in vivo*" at 16-20 °C (Valkiūnas, 2005; Valkiūnas et al., 2013; Žiegytė et al., 2014b). During the normal life cycle, *Haemoproteus* spp. ookinetes migrate through the epithelial layer of the midgut of the vector and round up under the basal lamina giving rise to oocysts (Valkiūnas, 2005). Our study shows that *Haemoproteus* spp. ookinetes can rapidly penetrate through the midgut epithelial layer, reach the haemocoel, and migrate in haemolymph forming large clumps of parasites (Fig. 1 in **Paper III**), which most likely interrupt haemolymph circulation and probably damage mechanically organs in abdomen and thorax of biting midges. Available experimental studies show that high parasitaemia with *H. tartakovskyi* and *H. balmorali* are lethal for *Ocherotatus cantans* and probably for other bird-biting mosquitoes. Ookinetes were reported in abdomen, thorax and head of infected mosquitoes (Valkiūnas et al., 2013). We show that the same is true for *Culicoides* biting midges infected with *H. lanii*. Additionally, mortality is even more severe in tiny biting midges, which rapidly die before ookinetes reach the head.

This study and earlier experimental research (Liutkevičius, 2000; Valkiūnas and Iezhova, 2004; Valkiūnas, 2005) showed that high *Haemoproteus* spp. gametocytaemia is responsible for mortality in biting midges. It is thus probable that lower blood meal parasitaemia (0.1% or less) is preferable for effective *Haemoproteus* parasite transmission in wildlife.

3.6. Sporogonic development of avian malaria parasites in laboratory-reared mosquitoes

In **Paper II,** we compared the sporogonic development of *Plasmodium relictum* (GRW4) in experimentally infected *Culex pipiens* form *molestus* and *C. quinquefasciatus* mosquitoes.

Ookinetes, oocysts, and sporozoites were reported in both species of insects on the same day after infection. In other words, the pattern of sporogonic development of this malaria strain was the same in these mosquito species. Morphologically similar sporogonic stages developed in *C. pipiens* form *molestus* and *C. quinquefasciatus*. There were no discernible morphometric differences among mature ookinetes or sporozoites, which developed in different mosquito species ($P>0.2$ for all corresponding data). However, significantly larger mature oocysts were seen in *C. quinquefasciatus* ($P<0.01$ for oocysts' area) (Fig. 4 in **Paper II**).

Culex quinquefasciatus transmits the lineage pGRW4 of *P. relictum* (Atkinson et al., 2008; LaPointe et al., 2010), which has been reported to be actively transmitted mainly in countries with warm climates (Ricklefs et al., 2004; Beadell et al., 2006; Bensch et al. 2009; Marzal et al., 2011; Loiseau et al., 2012). The sporogony completed and numerous sporozoites appeared in the salivary glands both of *C. pipiens* form *molestus* and *C. quinquefasciatus* mosquitoes at relatively low temperature conditions, which are close to the long-term mean air degrees reported in the majority of European regions with temperate climates during the warmest months of a year (Earth System Research Laboratory, Colorado; Web site at http:// www.esrl.noaa.gov/psd/) when active transmission of malaria parasites occurs (Valkiūnas, 2005). Earlier experimental studies also reported the complete sporogony of this parasite in *C. quinquefasciatus* at the same temperatures (LaPointe et al., 2010). Because *C. pipiens* form *molestus* mosquito is widespread in the Holarctic (Vinogradova, 2000; Gomes et al., 2009) and sporogony of *P. relictum* (pGRW4) successfully completes at a mean temperature of 19 °C in this mosquito 16 dpi, we conclude that there are no restrictions for spreading of this avian malaria infection in temperate regions of Europe from the point of view of vector availability and air temperature necessary for sporogony.

In **Paper IV,** we examined the sporogonic development of a new species of *Plasmodium* genus, *P. delichoni* (pCOLL6). Experimentally infected mosquitoes *C. pipiens* form *molestus*, *C. quinquefasciatus* and *A. aegypti* were resistant. Numerous remnants of blood stages were seen in mosquito midgut contents of each species, and a few degrading ookinetes were observed in mosquitoes *Aedes aegypti* and *C. pipiens* form *molestus*. Oocysts did not develop and sporozoites were not seen in salivary glands. Sporogonic development is abortive on gametogenesis or ookinete stages in all exposed mosquito species.

This study showed the lack of sporogonic development in mosquitoes which are widespread in Europe (*C. pipiens* form *molestus*) and sub-Saharan Africa (*C. quinquefasciatus*, *A. aegypti*). Even gametogenesis and development of ookinetes were abortive in these mosquitoes, indicating that the sexual process, ookinete formation and sporogony of *P. delichoni* require particular mosquito gut factors (pH) for successful development in vectors, as is the case in human malaria parasites (Sherman 1998). It is probable that the lack of specific mosquito vectors is an important factor preventing the spreading of *P. delichoni* infection in Europe. Endemic African mosquitoes should be tested for their vectorial capability. The sporogony of avian *Plasmodium* spp. completes and sporozoites develop in *Coquillettidia* mosquitoes transmitting avian malaria in Africa (Njabo et al.,

2009). The species of this genus and related genera of bird-biting Culicidae should be considered in future experimental vector research in avian malaria studies. Such research is important for a better understanding of the mechanisms preventing this disease spreading by migrating birds from the tropics to areas with temperate climates. *Plasmodium delichoni* is a convenient model parasite for such research because it develops long-lasting parasitemia in domestic canaries. These birds are easy to breed, maintain and infect in captivity, and they can be used as donors of gametocytes for experimental infection to different mosquito species.

FOR MORE DETAILED RESULTS AND DISCUSSION SEE PAPERS I-V

CONCLUSIONS

1. An easy to use method for the experimental infections of laboratory-reared biting midges *Culicoides nubeculosus* with avian *Haemoproteus* parasites was developed.
2. The sporogonic development of *Haemoproteus noctuae* completes in experimentally infected biting midges *Culicoides nubeculosus* and *C. impunctatus,* and *H. syrnii* completes sporogony in *C. nubeculosus*. These insects are likely natural vectors.
3. The sporozoites of *Haemoproteus noctuae* and *H. syrnii* are readily distinguishable by size, suggesting possible application of this character in identification of these parasites in vectors.
4. Three species of *Haemoproteus* (*H. minutus, H. motacillae,* and *H. attenuatus)* of passeriform birds complete sporogonic development in laboratory-reared biting midges *Culicoides nubeculosus*, and this insect is likely a natural vector.
5. Phylogenies based on partial *cytb* gene readily indicate groups of possible vectors of avian haemoproteids. Analysis of such phylogenies is recommended before designing experimental studies with insects.
6. Blood meal with high *Haemoproteus lanii* gametocytaemia leads to high mortality of biting midges *Culicoides impunctatus* due to the damage of abdomen and thorax by migration parasite ookinetes.
7. Ther sporogony of *Plasmodium relictum* (lineage pGRW4) completes in mosquitoes *Culex pipiens* form *molestus* and *C. quinquefasciatus* at relatively low temperatures, indicating the lack of obstacles for this parasite transmission in Europe in regard of the availability of vector and temperature conditions.
8. The sporogonic development of *Plasmodium delichoni* (pCOLL6) is abortive in experimentally infected mosquitoes *Culex pipiens* form *molestus*, *C. quinquefasciatus,* and *Aedes aegypti*.

SCIENTIFIC AND PRACTICAL SIGNIFICANCE

1. We suggest an easy to use method for experimental research on the sporogonic development of avian haemoproteids in laboratory-reared biting midges *Culicoides nubeculosus*. This provides new opportunities for experimental research with *Haemoproteus* species.
2. *Culicoides nubeculosus* supports complete sporogony of many avian *Haemoproteus* species. This insect is easy to rear in laboratory conditions and is recommended for experimental research with avian haemoproteids.
3. Phylogenies based on partial *cytb* gene readily indicate groups of possible vectors of avian haemoproteids (biting midges or louse flies); they are easy to construct and are recommended to analyze before designing experimental studies with insects.
4. Due to high mortality of biting midges during heavy *Haemoproteus* infections, we recommend using parasitemias of approximately 0.1% in experimental sporogonic studies of haemoproteids.
5. The vectors of pathogenic malaria parasite *Plasmodium relictum* (lineage pGRW4) are available in Europe, and this should be taken into consideration in epidemiological studies of this avian malaria infection.

SANTRAUKA

MOKSLINĖ PROBLEMA

Hemosporidiniai parazitai, priklausantys *Haemoproteus* ir *Plasmodium* (Haemosporida, Haemoproteidae) gentims, yra svarbūs paukščių patogenai, plačiai paplitę daugelyje paukščių populiacijų, sukeliantys ligas bei dažnai tampantys paukščių mirtingumo priežastimi (Garvin ir kt., 2003; Donovan ir kt., 2008; Olias ir kt., 2011; Pacheco ir kt., 2011; Palinauskas ir kt., 2015). Esama daug informacijos apie paukščių hemosporidinių parazitų paplitimą (Silva-Iturriza ir kt., 2012; Ishtiaq ir kt., 2007; Latta ir Ricklefs, 2010), genetinę įvairovę (Dimitrov ir kt., 2010; Belo ir kt., 2011; Szymanski ir Lovette, 2005; Ivanova ir kt., 2015) ir filogenetinius ryšius (Santiago-Alarcon ir kt., 2010; Carlson ir kt., 2013; Bensch ir kt., 2016; Yoshimura ir kt., 2014), tačiau šių kraujo parazitų pernešėjai, sporogoninis vystymasis bei transmisijos modeliai yra nepakankamai ištirti. Tai yra pagrindinės kliūtys, trukdančios geriau suprasti šių patogenų sukeliamų ligų epidemiologiją, prevencinių priemonių rengimą ir tobulinimą (**V straipsnis**).

Haemoproteus genties parazitus perduoda briedmusės (Hippoboscidae) ir smulkieji mašalai (Ceratopogonidae). *Parahaemoproteus* pogentės rūšis perneša smulkieji mašalai (*Culicoides*) (Garnham, 1966; Valkiūnas, 2005; Atkinson ir kt., 2008). Naujausi eksperimentiniai tyrimai parodė, kad plačiai paplitęs smulkusis mašalas *Culicoides impunctatus* yra imlus daugeliui *Parahaemoproteus* pogentės rūšių (Žiegytė ir kt., 2017), tačiau, dėl sudėtingų eksperimentinių tyrimų, atliekamų su šiais vabzdžiais, yra labai mažai informacijos apie kitų pernešėjų imlumą, nes jų gausos laikotarpis gamtoje yra labai trumpas (keletas savaičių pavasario-vasaros metu) (Liutkevičius, 2000). Be to, dėl didelio šių laukinių smulkiųjų mašalų mirtingumo nelaisvėje, eksperimentiniai tyrimai tampa sudėtingesni (Valkiūnas ir Iezhova, 2004). Laboratorijoje auginamų smulkiųjų mašalų sporogonijos eksperimentiniai tyrimai būtų naudingi, tačiau dar nepakankamai parengta šio tyrimo metodika. Kita *Culicoides* rūšis (*C. nubeculosus*) yra plačiai paplitusi Europoje (Mathieu ir kt., 2012). Šis vabzdys buvo kolonizuotas (Boorman, 1974) ir yra imlus kelioms *Haemoproteus* genties rūšims (Miltgen ir kt., 1981; Žiegytė ir kt., 2016). Vis dėlto lieka neaišku, kurie *Haemoproteus* genties parazitai gali užbaigti sporogoniją *C. nubeculosus* mašaluose. Svarbu paminėti, kad, siekiant identifikuoti pernešėją, yra būtina aptikti sporozoitus vabzdyje, nes galimo

abortyvaus vystymosi PGR (polimerazės grandinine reakcija) paremti metodai nenustato (Valkiūnas ir kt., 2013) (**I ir V straipsniai**).

Buvo atlikta daug tyrimų, kuriais nustatytas sumažėjęs laukinių paukščių, užsikrėtusių *Haemoproteus* genties parazitais, išgyvenamumas, taip pat pastebėtas neigiamas poveikis imuninei sistemai, kūno būklei ir reprodukcijai (Marzal ir kt., 2005; Valkiūnas ir kt., 2006; La Puente ir kt., 2010). Tačiau apie *Haemoproteus* infekcijų poveikį kraujasiurbiams vabzdžiams žinoma mažai. Eksperimentiniai tyrimai parodė, kad aukšta hemoproteidų gametocitemija yra mirtina *Culicoides* genties mašalams. Liutkevičius (2000), bei Valkiūnas ir Iezhova (2004) pažymėjo, kad nustatytas, didelis *Hemoproteus balmorali*, *H. belopolskyi*, *H. dolniki*, *H. fringillae*, *H. lanii* ir *H. tartakovskyi* parazitais infekuotų *C. impunctatus* mašalų mirtingumas, tačiau mirtingumo mechanizmai išlieka neaiškūs. Buvo manoma (Valkiūnas ir Iezhova, 2004), kad smulkiųjų mašalų mirtingumą galėjo sukelti ookinečių ir/arba oocistų vystymasis, kuris pažeidžia vidurinės žarnos epitelines ląsteles ir gali sukelti uždegimines reakcijas, tačiau smulkiųjų mašalų histologiniai stebėjimai neatlikti (**III straipsnis**).

Plasmodium genties rūšys sukelia paukščių maliariją (Garnham, 1966; Valkiūnas ir kt., 2008; Mantilla ir kt., 2013; Ilgūnas ir kt., 2013; Walther ir kt., 2014). Iš viso yra aprašytos 55 *Plasmodium* rūšys, užkrečiančios paukščius (Valkiūnas ir Iezhova, 2018), tačiau šių mirtinų parazitų pernešėjai rūšies lygyje yra nepakankamai ištirti.

Plasmodium relictum (Haemosporida, Plasmodiidae) sukelia paukščių maliariją ir yra dažniausiai aptinkamas paukščių maliarinis parazitas užkrečiantis daugiau nei 300 paukščių rūšių visuose žemynuose, išskyrus Antarktiką (Garnham, 1966; Valkiūnas, 2005; Atkinson ir kt., 2008; Valkiūnas ir kt., 2018). Dvi *P. relictum* linijos (pSGS1 ir pGRW4), kurios yra plačiai paplitusios tiek geografiškai tiek šeimininkuose (Beadell ir kt., 2006; Ejiri ir kt., 2009; Clark ir kt., 2014; Perkins, 2014), aptinkamos viso pasaulio paukščiuose, tačiau jų transmisijos teritorija skiriasi (Marzal ir kt., 2011). Linija pSGS1 yra perduodama visame pasaulyje (Bensch ir kt., 2009; Marzal ir kt., 2015). *Culex pipiens* uodai yra efektyvūs šių parazitų pernešėjai (Vézilier ir kt., 2010; Kazlauskienė ir kt., 2013) sporogonija juose baigiasi esant 12-30 °C temperatūrai (Garnham, 1966; Valkiūnas, 2005; Žiegytė ir kt., 2014a). Tuo tarpu, linija pGRW4 aktyviai plinta daugiausia šilto klimato šalyse (Ricklefs ir kt., 2004; Beadell ir kt., 2006; Bensch ir kt., 2009; Marzal ir kt., 2011; Loiseau ir kt., 2012). *Culex quinquefasciatus* taip pat yra efektyvus šių parazitų pernešėjas (Atkinson ir kt., 2008; LaPointe ir kt., 2010; Freed and Cann, 2013), tačiau šio vabzdžio nėra vidutinio klimato juostoje Europoje, kur *C. pipiens* uodai yra paukščių maliarijos pernešėjai

(Vézilier ir kt., 2010; Kazlauskienė ir kt., 2013; Cornet ir kt., 2013). Lieka neaišku ar linija pGRW4 gali užbaigti sporogoniją *C. pipiens* forma *molestus* ar kituose Europoje plačiai paplitusiuose uoduose ir ar tai vyksta santykinai žemoje temperatūroje. Šiame tyrime nagrinėjamas pastarasis klausimas (**II straipsnis**).

Naujas mokslui maliarinis parazitas *Plasmodium* (Novyella) *delichoni* (Haemosporida, Plasmodiidae) buvo neseniai aptiktas (Valkiūnas ir kt., 2016) plačiai paplitusioje naminėje kregždėje *Delichon urbicum* (Hirundinidae). Kadangi šio parazito pernešėjai yra nežinomi, būtina juos nustatyti (**IV straipsnis**).

TIKSLAS IR UŽDAVINIAI

Šio darbo tikslas – ištirti plačiai paplitusių *Haemoproteus* ir *Plasmodium* genties rūšių parazitų sporogoninį vystymąsi laukinių ir laboratorijoje auginamų kraujasiurbių vabzdžų organizmuose bei nustatyti šių parazitų pernešėjus.

Tikslui įgyvendinti buvo iškelti tokie uždaviniai:

1. Sukurti *Culicoides nubeculosus* smulkiųjų mašalų užkrėtimo *Haemoproteus* genties parazitais metodą.
2. Ištirti sporogoninį vystymąsi ir nustatyti *Haemoproteus* genties pelėdų parazitų rūšių pernešėjus.
3. Ištirti sporogoninį vystymąsi ir nustatyti žvirblinių paukščių trijų *Haemoproteus* parazitų pernešėjus.
4. Nustatyti *Haemoproteus* genties parazitų, kurių pernešėjai identifikuoti, filogenetinius ryšius.
5. Ištirti *Haemoproteus lanii* parazitu užkrėstų *Culicoides impunctatus* mašalų mirtingumo mechanizmus.
6. Nustatyti ar *Plasmodium relictum* (linija pGRW4) ir *P. delichoni* sporogonija vyksta laboratorijoje auginamų uodų organizmuose.

GINAMIEJI TEIGINIAI

1. Mūsų sukurtas *Culicoides nubeculosus* smulkiųjų mašalų užkrėtimo *Haemoproteus* genties parazitais metodas yra tinkamas eksperimentiniams darbams.
2. Dvi pelėdų parazitų (*Haemoproteus noctuae* ir *H. syrnii*) rūšys užbaigia sporogoninį vystymąsi laboratorijoje auginamų smulkiųjų

mašalų *Culicoides nubeculosus* ir gyvenančių gamtoje *C. impunctatus* organizmuose. Šie vabzdžiai yra galimi pernešėjai.

3. Trijų žvirblinių būrio paukščių *Haemoproteus* parazitų sporogonija vyksta *Culicoides nubeculosus* mašalų organizmuose ir šis vabzdys yra jų pernešėjas.
4. Filogenetiniai tyrimai, paremti mitochondrinio citochromo *b* (*cytb*) genu, parodo paukščių hemoproteidų pernešėjų grupes.
5. Migruojančios ookinetės, bet ne oocistos yra pagrindinė smulkiųjų mašalų, užkrėstų aukšta *Haemoproteus lanii* gametocitemija, mirties priežastis.
6. *Plasmodium relictum* (linija pGRW4) užbaigia sporogoninį vystymąsi laboratorijoje auginamuose *Culex quinquefasciatus* ir *C. pipiens* forma *molestus* uoduose, o *P. delichoni* nesivysto *C. quinquefasciatus*, *C. pipiens* forma *molestus* ir *Aedes aegypti* uodų organizmuose.

DARBO NAUJUMAS

1. Buvo sukurtas lengvai pritaikomas metodas laboratorijoje auginamiems smulkiesiems mašalams (*Culicoides nubeculosus*) užkrėsti. Naudojant šį metodą buvo nustatyta, kad penkios *Haemoproteus* genčiai priklausančios parazitų rūšys (*H. noctuae*, *H. syrnii*, *H. minutus*, *H. motacillae* ir *H. attenuatus)* užbaigia sporogoniją šio mašalo organizme. Taip pat, buvo nustatyta, kad *cytb* genu paremti filogenetiniai tyrimai parodo galimus hemoproteidų pernešėjus. Tai buvo paaiškinta pirmą kartą.
2. Buvo nustatyta, kad aukštos *Haemoproteus* infekcijos yra mirtinos smulkiesiems mašalams dėl pažeidimų, kuriuos pilvelyje ir krūtinėlėje sukelia migruojančios ookinetės.
3. Šis tyrimas pirmą kartą parodė, kad *Plasmodium relictum* (linija pGRW4) užbaigia sporogoniją *Culex pipiens* forma *molestus* ir *C. quinquefasciatus* uodų, su panašiomis vystymosi ypatybėmis, organizmuose. Tuo tarpu *P. delichoni* vystymasis *C. pipiens* forma *molestus, C. quinquefasciatus, Aedes aegypti* uodų organizmuose yra abortyvus.

LITERATŪROS APŽVALGA

Literatūros apžvalgoje glaustai pateikta informacija apie hemosporidinių parazitų istoriją, gyvybinius ciklus, pernešėjus ir molekulinius metodus.

MEDŽIAGA IR METODAI

Išsamus metodų aprašymas yra pateiktas tekste nurodytuose straipsniuose.

Eksperimentinių tyrimų metu buvo gaudomi paukščiai, imamas jų kraujas molekuliniams tyrimams (fiksuojama SET buferyje) ir daromi kraujo tepinėliai (**I-V straipsniai**).

Eksperimentiniuose tyrimuose buvo naudojami laukiniai ir laboratorijoje auginami kraujasiurbiai vabzdžiai. Laboratorijoje buvo kultivuojama *Culicoides nubeculosus* smulkiųjų mašalų kolonija, jie buvo naudojami eksperimentiniams hemosporidinių parazitų vystymosi (**I** ir **V straipsniai**) tyrimams. Laukiniai smulkieji mašalai *C. impunctatus* buvo naudojami eksperimentiniams hemosporidinių parazitų vystymosi (**I straipsnis**) ir mirtingumo (**V straipsnis**) tyrimams. Laboratorijoje kultivuojamos kraujasiurbių uodų kolonijos (*Culex quinquefasciatus*, *C. pipiens* forma *molestus* ir *Aedes aegypti*) buvo naudojamos atliekant eksperimentinius parazito vystymosi (**II** ir **IV straipsniai**) užkrėtimus.

Visi eksperimento būdu užkrėsti vabzdžiai buvo laikomi kontroliuojamomis sąlygomis (fotoperiodas, drėgmė, temperatūra) ir skrodžiami tam tikromis valandomis ir dienomis, ieškoma parazito vystymosi stadijų (**I-V straipsniai**).

Siekiant nustatyti parazito rūšį ir patvirtinti parazito buvimą vabzdyje (**I-V straipsniai**) buvo naudojami molekuliniai metodai.

Statistinė analizė buvo atlikta naudojantis „Statistica 7“ (**I-III straipsniai**) ir „R studio“ versijos 3.4.3 (**V straipsnis**) programomis. Siekiant palyginti nepriklausomas imtis buvo naudojamas Studento t-testas. Išgyvenamumo procentai tarp kontrolinės ir eksperimentinės grupių buvo palyginti naudojant Fišerio tikslųjį testą. Jeigu P reikšmė 0.05 arba mažiau, buvo laikoma, kad skirtumas reikšmingas.

Eksperimentų metu visi laukiniai paukščiai išgyveno ir buvo paleisti į laisvę.

REZULTATAI IR APTARIMAS

Kolonizuotų *Culicoides nubeculosus* mašalų užkrėtimo *Haemoproteus* paukščių parazitais eksperimento būdu, metodo kūrimas

Miltgen ir kt. (1981) atliekant eksperimentą užkrėtė *Culicoides nubeculosus* smulkiuosius mašalus *Haemoproteus handai* (sin. *H. desseri*) parazitu ir pirmasis parodė sporozoitų vystymąsi, tačiau eksperimento eiga straipsnyje nebuvo aprašyta. Mes išvystėme šį metodą ir išsamiai aprašėme **I** ir **V straipsniuose**. Šį metodą yra paprasta naudoti atliekant eksperimentinius smulkiųjų mašalų užkrėtimus *Haemoproteus* parazitais.

Pelėdų hemosporidinių parazitų sporogonija smulkiuosiuose mašaluose

Mes ištyrėme ir nustatėme, kad du pelėdų parazitai užbaigia sporogoniją eksperimento metu užkrėstuose smulkiuosiuose mašaluose (**I straipsnis**). *Haemoproteus noctuae* ookinetės, oocistos ir sporozoitai sėkmingai išsivystė laukiniuose *Culicoides impunctatus* ir laboratorijoje auginamuose *C. nubeculosus* smulkiuosiuose mašaluose. Tuo tarpu *H. syrnii* užbaigė sporogoniją *C. nubeculosus* mašaluose. *Haemoproteus noctuae* skirtingų mašalų rūšių sporozoitų plotis, plotas ir branduolio ilgis bei plotas reikšmingai nesiskyrė ($P > 0.05$). Taip pat *H. syrnii* sporozoitai buvo reikšmingai trumpesni ir mažesni pagal plotą, lyginant su *H. noctuae* sporozoitais ($P < 0.05$).

Abu šie parazitai užbaigia sporogoniją *Culicoides* mašaluose, kaip ir kiti *Parahaemoproteus* pogenčiai priklausantys parazitai (Atkinson ir kt., 1988; Valkiūnas, 2005; Žiegytė ir kt., 2014b) ir, tikėtina, yra natūralūs pernešėjai. Siekiant įrodyti, kad vabzdžiai gali būti pernešėjais, yra būtina seilių liaukose aptikti sporozoitus (Žiegytė ir kt., 2014b; Valkiūnas ir kt., 2002; Atkinson, 1991; Fallis ir Wood, 1957).

Žvirbliniuose paukščiuose plačiai paplitusių trijų hemosporidinių parazitų sporogonija laboratorijoje auginamuose *Culicoides nubeculosus* smulkiuosiuose mašaluose

Mes ištyrėme ir nustatėme (**V straipsnis**), kad žvirblinių paukščių trijų hemosporidinių parazitų sporogonija vyksta eksperimento būdu užkrėstuose *Culicoides nubeculosus* mašaluose. *Haemoproteus minutus*, *H. motacillae* ir *H. attenuates* sporozoitai išsivystė ir buvo matomi seilių liaukų

preparatuose (žiūrėti 1 pav. m-o, **V straipsnis**). Remiantis morfologiniais požymiais, skirtingų rūšių sporozoitai *C. nubeculosus* mašaluose skiriasi.

Turimi duomenys rodo, kad *C. nubeculosus* mašaluose vystosi iš viso septynios hemoproteidų rūšys ir šie mašalai yra jų pernešėjai. Kitaip tariant, šis vabzdys yra labai imlus daugeliui *Haemoproteus* infekcijų ir, tikėtina, dalyvauja gamtoje vykstančioje hemoproteidų transmisijoje. Taip pat ir laukiniai *C. impunctatus* mašalai buvo naudojami eksperimentiniuose tyrimuose (Žiegytė ir kt., 2014b; Žiegytė ir kt., 2017). Tačiau, eksperimentinis darbas su laukiniais *C. impunctatus* mašalais yra sudėtingas dėl trumpo jų gausos periodo (keletas savaičių pavasario-vasaros metu), todėl, atliekant užkrėtimo eksperimentus, sudėtinga surinkti pakankamą mašalų kiekį (Liutkevičius, 2000). Dėl to tampa sunkiau naudoti šiuos vabzdžius eksperimentiniuose darbuose. Be to, yra didelis laukinių *C. impunctatus* mašalų mirtingumas nelaisvėje (Valkiūnas and Iezhova, 2004). Tuo tarpu, *C. nubeculosus* lengva auginti ir jie gana gerai gyvena laboratorijoje (Boorman, 1974). Mes rekomenduojame naudoti šį mašalą atliekant eksperimentinius *Haemoproteus* genties parazitų tyrimus, kuriuos galima vykdyti ištisus metus. Tai atveria naujas galimybes stebėti hemosporidinius parazitus įvairiais biologijos aspektais eksperimentų metu.

Culicoides genties rūšių, pernešančių *Haemoproteus* genties parazitus, filogenetinių ryšių analizė

Visos šiame tyrime naudotos *Haemoproteus* rūšys (**I ir V straipsniai**) grupuojasi kartu su kitais *Culicoides* pernešamais parazitais. Šie parazitai priklauso *Parahaemoproteus* pogenčiai. Pogenčiai *Haemoproteus* priklausančios rūšys, kurias perneša briedmusės, patenka į atskirą patikimą šaką (4 pav., **I straipsnis**).

Remdamiesi gautais rezultatais, mes spėjame, kad visų hemoproteidų pernešamų mašalų *cytb* linijos grupuosis kartu, tuo tarpu briedmusių pernešamų parazitų linijos grupuosis kartu kitoje šakoje. Kitaip tariant, *cytb* sekų filogenetinė analizė leidžia nuspėti hemoproteidų parazitų pernešėjų grupes (Ceratopogonidae arba Hippoboscidae).

Santykinai griežtos tam tikros hemosporidinių parazitų šakų sąsajos su konkrečiomis vabzdžių šeimomis nėra pakankamai suprastos ir įtikinamai paaiškintos. Tai gali reikšti, jog hemosporidinių parazitų vystymasis pernešėjuose gali būti labai svarbus šių parazitų mtDNR genų evoliucijai. Savo gyvenimo ciklo metu šie protistai naudoja ir glikolizę, ir oksidacinį fosforilinimą. Nors glikolizė dominuoja hemosporidijų vystymosi stuburiniame šeimininke metu, parazitas pereina prie oksidacinio

fosforilinimo proceso pernešėjuje, kuriame gliukozė nepakankamai pasiekiama adenozintrifosfato sintezei (Hino ir kt., 2012). Taigi, mitochondriniai genai yra svarbūs hemosporidijų išgyvenamumui pernešėjuose, bet jų vystymasis stuburiniuose, kuriuose dominuoja glikolizė yra ne tiek svarbus (Hall ir kt., 2005; Jacot ir kt., 2016; Pacheco ir kt., 2018). Filogenijos, paremtos mtDNR, gali gerai atspindėti parazito-pernešėjo evoliucinius ryšius, bet ne taip gerai evoliucijos modelių - stuburinis šeimininkas-parazitas-pernešėjas, sistemas. Tuo galima paaiškinti didelius skirtumus tarp skirtingais genais paremtų filogenijų (Bensch ir kt., 2016). Siekiant atsakyti į šį klausimą, reikia išsamiai išanalizuoti visą hemosporidijų genomą.

Culicoides impunctatus smulkiųjų mašalų, užkrėstų *Haemoproteus* infekcijomis, mirtingumo mechanizmai

Mes nustatėme (**III straipsnis**) laukinių mašalų *Culicoides impunctatus*, užkrėstų *Haemoproteus lanii* aukšta gametocitemija (5.2%) išgyvenamumą ir mirtingumo mechanizmus. Kontrolinės ir eksperimentinės grupių išgyvenamumas reikšmingai skiriasi (Fišerio tikslusis testas, $P < 0.001$). Dauguma užkrėstų mašalų (98%) buvo negyvi jau 12 valandų po užkrėtimo. Daug ookinečių buvo aptikta vidurinės žarnos turinio ir histologiniuose krūtinėlės ir pilvelio preparatuose (1 pav., **III straipsnis**).

Svarbiausias šio eksperimento rezultatas yra tas, kad pagrindinė mašalų mirties priežastis yra migruojančios ookinetės, kurios prasiskverbia pro vidurinės žarnos sienelę, ją pažeidžia, kaupiasi hemocelyje ir sutrikdo hemolimfos cirkuliaciją, taip pat gali mechaniškai pažeisti organus, esančius pilvelyje ir krūtinėlėje.

Skirtingų *Haemoproteus* genties rūšių ookinetės subręsta 2-12 valandą po užkrėtimo atliekant "*in vitro*" ir "*in vivo*" eksperimentus, esant 16-20 °C temperatūrai (Valkiūnas, 2005; Valkiūnas ir kt., 2013; Žiegytė ir kt., 2014b). Įprastai gyvenimo ciklo metu *Haemoproteus* ookinetės migruoja pro vidurinės žarnos epitelinį sluoksnį, po bazaline membrana suapvalėja ir pradeda vystytis oocistos (Valkiūnas, 2005). Mūsų tyrimas rodo, kad *Haemoproteus* spp. ookinetės greitai prasiskverbia pro vidurinės žarnos epitelinį sluoksnį, pasiekia hemocelį, migruoja hemolimfa, suformuodamos santalkas (1 pav., **III straipsnis**), kurios sutrikdo hemolimfos cirkuliaciją ir, veikiausiai, mechaniškai pažeidžia vabzdžio pilvelio ir krūtinėlės organus. Eksperimentiniai tyrimai rodo, kad aukšta *H. tartakovskyi* ir *H. balmorali* gametocitemija yra mirtina *Ocherotatus cantans* uodams ir, tikriausiai, kitiems paukščių kraują siurbiantiems uodams. Užkrėstų uodų pilvelyje,

krūtinėlėje ir galvoje buvo aptiktos ookinetės (Valkiūnas ir kt., 2013). Mes parodome, kad tie patys procesai vyksta ir *Culicoides* smulkiuosiuose mašaluose, užkrėstuose *H. lanii* parazitu. Be to, smulkiųjų mašalų mirtingumas yra didesnis ir jie miršta greičiau, nei ookinetės pasiekia galvą.

Šis tyrimas ir ankstesni eksperimentai (Liutkevičius, 2000; Valkiūnas ir Iezhova, 2004; Valkiūnas, 2005) rodo, kad aukšta *Haemoproteus* spp. gametocitemija yra atsakinga už mašalų mirtingumą. Tikėtina, kad žema gametocitemija (0.1% ir mažiau) yra būtina efektyviam *Haemoproteus* parazitų perdavimui gamtoje.

Paukščių maliarinių parazitų sporogoninis vystymasis laboratorijoje auginamuose uoduose

II straipsnyje mes palyginome *Plasmodium relictum* (GRW4) vystymąsi eksperimento būdu užkrėstuose *Culex pipiens* forma *molestus* ir *C. quinquefasciatus* uoduose.

Tomis pačiomis dienomis po užkrėtimo buvo aptiktos abiejų rūšių ookinetės, oocistos ir sporozoitai. Kitaip tariant, šis parazitas panašiai vystosi skirtingose uodų rūšyse. *Culex pipiens* forma *molestus* ir *C. quinquefasciatus* uoduose išsivystė morfologiškai panašios parazito stadijos. Nebuvo aptikta jokių pastebimų morfometrinių skirtumų ookinečių ir sporozoitų stadijose, kurios išsivystė skirtingose uodų rūšyse ($P>0.2$ visiems matavimams). Tačiau *C. quinquefasciatus* uoduose buvo matomos reikšmingai didesnės oocistos ($P<0.01$) (4 pav., **II straipsnis**).

Culex quinquefasciatus perneša pGRW4 parazitą (Atkinson ir kt., 2008; LaPointe ir kt., 2010). Buvo nustatyta, kad *P. relictum* linija pGRW4 aktyviai perduodama daugiausia šilto klimato šalyse (Ricklefs ir kt., 2004; Beadell ir kt., 2006; Bensch ir kt., 2009; Marzal ir kt., 2011; Loiseau ir kt., 2012). Sporogonija buvo sėkmingai užbaigta ir sporozoitai buvo rasti *C. pipiens* forma *molestus* ir *C. quinquefasciatus* uodų seilių liaukose prie sąlyginai nedidelės temperatūros. Ši temperatūra yra artima daugiamečių temperatūros stebėjimų vidurkiui šilčiausiais metų mėnesiais (Earth System Research Laboratory, Colorado; Web site at http:// www.esrl.noaa.gov/psd/), tuo metu, kai maliariniai parazitai yra aktyviai perduodami (Valkiūnas, 2005), daugelyje švelnaus klimato Europos regionų. Ankstesni eksperimentiniai tyrimai taip pat nustatė sporogoninį vystymąsi *C. quinquefasciatus* uoduose tokioje pačioje temperatūroje (LaPointe ir kt., 2010). Kadangi *C. pipiens* forma *molestus* uodai yra paplitę Holarktikoje (Vinogradova, 2000; Gomes ir kt., 2009) ir *P. relictum* (pGRW4) sporogonija sėkmingai užsibaigia 16 dieną po užkrėtimo esant 19 °C

temperatai. Mes darome išvadą, kad vidutinio klimato juostoje nėra jokių apribojimų šio parazito plitimui temperatūros ir pernešėjų atžvilgiu.

IV straipsnyje mes ištyrėme naujo mokslui *Plasmodium* genties parazito, *P. delichoni* (pCOLL6), sporogoninį vystymąsi. Eksperimento būdu užkrėsti uodai *C. pipiens* forma *molestus*, *C. quinquefasciatus* ir *Aedes aegypti* buvo atsparūs šiam parazitui. Kiekvienos vabzdžio rūšies pilvelyje, buvo matoma daug parazito kraujo stadijų liekanų, taip pat buvo aptiktos kelios degraduojančios ookinetės *A. aegypti* ir *C. pipiens* forma *molestus* uoduose. Oocistos neišsivystė ir sporozoitai nebuvo aptikti seilių liaukose. Visose uodų rūšyse sporogoninis vystymasis gametogenezės arba ookinetės stadijoje yra abortyvus.

Šis tyrimas parodė, kad uoduose, paplitusiuose Europoje (*C. pipiens* forma *molestus*) ir Afrikoje į pietus nuo Sacharos (*C. quinquefasciatus*, *A. aegypti*), sporogonija nevyksta. Net gametogenezė ir ookinečių vystymasis šiuose uoduose buvo abortyvūs. Tikėtina, kad specifinių uodų pernešėjų trūkumas yra svarbus veiksnys, neleidžiantis plisti *P. delichoni* infekcijai Europoje. Dėl galimybės būti pernešėjais turėtų būti patikrinti endeminiai Afrikos uodai. Paukščių *Plasmodium* spp. sporogonija vyksta ir sporozoitai išsivysto *Coquillettidia* uoduose, kurie perneša paukščių maliariją Afrikoje (Njabo ir kt., 2009). Šios genties rūšys ir, susijusių genčių paukščių kraują siurbiantys *Culicidae* uodai, turėtų būti naudojami atliekant paukščių maliarinių parazitų tyrimus. Tokie moksliniai tyrimai yra svarbūs, siekiant geriau suprasti mechanizmus, užkertančius kelią šios ligos plitimui paukščių migravimo metu iš tropikų į vidutinio klimato juostą. Kadangi *P. delichoni* ilgai išsilaiko kanarėlėse, tai yra tinkamas parazito pavyzdys tokiems tyrimams. Šiuos paukščius yra lengva veisti, auginti ir užkrėsti nelaisvėje. Todėl jie gali būti naudojami kaip gametocitų donorai eksperimento būdu užkrečiant įvairias uodų rūšis.

IŠSAMŪS REZULTATAI IR APTARIMAS YRA PATEIKTI I-V STRAIPSNIUOSE

IŠVADOS

1. Buvo sukurtas ir išvystytas lengvai naudojamas metodas, kai smulkieji mašalai (*Culicoides nubeculosus*) ir *Haemoproteus* genties parazitai užkrečiami eksperimentiniu būdu.
2. Dviejų pelėdų parazitų (*Haemoproteus noctuae* ir *H. syrnii*) rūšių sporogonija užsibaigia eksperimento būdu užkrėstuose *Culicoides nubeculosus* ir *C. impunctatus* mašaluose, kurie yra galimi pernešėjai.
3. *Haemoproteus noctuae* ir *H. syrnii* sporozoitai skiriasi savo dydžiu. Siekiant identifikuoti šiuos parazitus pernešėjuose, galima remtis šiuo požymiu.
4. Trys žvirblinių paukščių *Haemoproteus* (*H. minutus, H. motacillae* and *H. attenuatus*) parazitų rūšys užbaigia sporogoniją laboratorijoje auginamuose *Culicoides nubeculosus* mašaluose ir šie vabzdžiai yra galimi pernešėjai.
5. Filogenetiniai tyrimai, paremti *cytb* genu, lengvai parodo galimų paukščių hemoproteidų pernešėjų grupes. Tokio tipo analizes rekomenduojama naudoti prieš planuojant eksperimentus su vabzdžiais.
6. Aukšta *Haemoproteus lanii* gametocitemija sukelia *Culicoides impunctatus* mašalų mirtingumą dėl migruojančių ookinečių, kurios pažeidžia pilvelį ir krūtinėlę.
7. *Plasmodium relictum* (pGRW4) sporogonija užsibaigia *Culex pipiens* forma *molestus* ir *C. quinquefasciatus* uoduose, santykinai žemoje temperatūroje - tai rodo, jog kliūtys šio parazito plitimui Europoje nėra susiję nei su pernešėjo nebuvimu nei su per žema temperatūra.
8. *Plasmodium delichoni* (pCOLL6) sporogoninis vystymasis yra abortyvus eksperimento būdu užkrėstuose *Culex pipiens* forma *molestus*, *C. quinquefasciatus* ir *Aedes aegypti* uoduose.

MOKSLINĖ IR PRAKTINĖ REIKŠMĖ

1. Mes siūlome lengvai naudojamą metodą, skirtą eksperimentiniams paukščių hemoproteidų sporogonijos tyrimams su laboratorijoje auginamais smulkiaisiais mašalais *Culicoides nubeculosus*. Tai atveria naujas eksperimentinių tyrimų su *Haemoproteus* genties parazitais galimybes.
2. Daugelis *Haemoproteus* genties parazitų užbaigia sporogoniją *Culicoides nubeculosus* mašaluose. Šį vabzdį lengva auginti laboratorijoje ir rekomenduojama naudoti eksperimentinių tyrimų su hemoproteidais metu.
3. Filogenetiniai tyrimai, paremti *cytb* genu, parodo galimas hemoproteidų pernešėjų grupes (smulkieji mašalai arba briedmusės); juos lengva sukonstruoti ir, prieš planuojant eksperimentinius tyrimus su vabzdžiais, rekomenduojama naudoti.
4. Dėl didelio smulkiųjų mašalų, užkrėstų aukštomis *Haemoproteus* gametocitemijomis, mirtingumo mes rekomenduojame eksperimentiniuose sporogonijos tyrimuose naudoti mažesnes negu 0.1% gametocitemijas.
5. Atliekant epidemiologinius paukščių maliarinių parazitų tyrimus reikėtų atsižvelgti į tai, kad maliarinio parazito *Plasmodium relictum* (linija pGRW4) pernešėjai aptinkami Europoje.

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Publications with an impact factor on the Clarivate Analytics Web of Science database.

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PAPER I

Biting midges (*Culicoides*, Diptera) transmit *Haemoproteus* parasites of owls: evidence from sporogony and molecular phylogeny

Bukauskaitė, D., Žiegytė, R., Palinauskas, V., Iezhova, T., Dimitrov, D., Ilgūnas, M., Bernotienė, R., Markovets, M.Yu., Valkiūnas, G.

Parasites & Vectors

2015, 8: 303

PAPER II

Complete sporogony of *Plasmodium relictum* (lineage pGRW4) in mosquitoes *Culex pipiens pipiens*, with implications on avian malaria epidemiology

Valkiūnas, G., Žiegytė, R., Palinauskas, V., Bernotienė, R., **Bukauskaitė, D.,** Ilgūnas, M., Dimitrov, D., Ježova, T.

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PAPER III

Mechanisms of mortality in *Culicoides* biting midges due to *Haemoproteus* infection

Bukauskaitė D., Bernotienė R., Iezhova T.A., Valkiūnas G.

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PAPER IV

***Plasmodium delichoni* n. sp.: description, molecular characterisation and remarks on the exoerythrocytic merogony, persistence, vectors and transmission**

Valkiūnas G., Ilgūnas M., **Bukauskaitė D.,** Žiegytė R., Bernotienė R., Jusys V., Eigirdas, V., Fragner K., Weissenbock H., Iezhova T.A.

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2016, 115, 2625-2636.

PAPER V

High susceptibility of the laboratory-reared biting midges *Culicoides nubeculosus* to *Haemoproteus* infections, with review on *Culicoides* species that transmit avian haemoproteids

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