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THE EXPERIMENTAL STUDY ON DEVELOPMENT OF AVIAN MALARIA PARASITES (*PLASMODIUM*) AND HAEMOPROTEIDS (*HAEMOPROTEUS*) IN VECTORS

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INTRODUCTION

Relevance of the study

Haemosporidian parasites (Haemosporida) belong to the group of haematozoa inhabiting many species of land vertebrates (amphibians, reptiles, birds, and mammals) almost all over the world. They use blood - sucking dipteran insects (Insecta: Diptera) as vectors (Valkiūnas, 2005; Telford, 2009; Perkins, 2014). Haemosporidians are a relatively well studied group of parasitic protists, particularly because they include the agents of malaria, which remains one of the common human diseases in countries with warm climate. Wildlife malaria parasites of the family Plasmodiidae have been used as model objects in research of this disease for over 100 years (Garnham, 1966; Perkins, 2014). Parasites of the family Haemoproteidae have been studied less, but certainly are important because they are markedly diverse and cause severe and even lethal diseases in some bird species (Miltgen et al., 1981; Atkinson et al., 1988; Cardona et al., 2002; Olias et al., 2011; Cannell et al., 2013). Many species of *Haemoproteus* (Haemoproteidae) and *Plasmodium* (Plasmodiidae) are responsible for acute and/or chronic diseases in many species of domestic and wild birds. These parasites are widespread in Europe, and are transmitted even in parks of large cities worldwide (Valkiūnas, 2005; Atkinson, 2008).

Haemosporidians of the genus *Plasmodium* cause malaria. Avian species of *Plasmodium* are common in birds at all continents except Antarctica (Valkiūnas, 2005; Bensch et al., 2009; Braga et al., 2011; Perkins, 2014). Species of *Haemoproteus* are also widespread in birds. Transmission of the representatives of both genera is particularly active in countries with warm and temperate climates, with overall infection prevalence exceeding 20 % in many terrestrial bird populations (Greiner et al., 1975; McClure et al., 1978; Peirce, 1981; Valkiūnas et al., 2003; Pérez-Tris et al., 2007; Shurulinkov and Ilieva, 2009). Malaria parasites and other haemosporidians often negatively affect both the vectors and vertebrate hosts (Merino et al., 2000; Palinauskas et al., 2008; Knowles et al., 2010; Kazlauskienė et al., 2013; Valkiūnas et al., 2014a).

Many recent studies addressed morphological and molecular characterization, distribution, genetic diversity and evolutionary relationships of avian *Haemoproteus* and *Plasmodium* spp. However, the information about relationships between bird haemosporidians and their vectors remains fragmentary and scarce (Kimura

et al., 2010). Knowledge about patterns of development of different haemosporidian species and their genetic lineages in vectors is insufficient (Ejiri et al., 2009; Kim et al., 2009; Kimura et al., 2010; LaPointe et al., 2010; Njabo et al., 2011). A few experimental studies deal with vectors and transmission of wildlife haemosporidian parasites (Atkinson, 1991b; Desser and Bennet, 1993; Valkiūnas et al., 2002; Kim et al., 2009; Levin et al., 2013). Little is known about the effects of avian haemosporidian infections on blood - sucking insects (Valkiūnas and Iezhova, 2004; Levin and Parker, 2014). These are obstacles for better understanding the epidemiology of diseases caused by avian haemosporidian parasites and the evolutionary biology of this large and diverse group of parasites.

Due to remarkable both genetic and phenotypic diversity, the cosmopolitan distribution and relative easy to sample and maintain under laboratory conditions, avian malaria parasites and haemoproteids are convenient objects to address important fundamental questions about pathogen evolutionary biology, ecology, emergence and many others issues. Information about genetic lineages of avian haemosporidians has been rapidly accumulating (Bensch et al., 2009; MalAvi – http://mbio-serv2.mbioekol.lu.se/Malavi/). However, insufficient knowledge about vectors prevents understanding epidemiology of the haemosporidioses, particularly on the level of their genetic lineages. Experimental research with avian haemosporidian parasites was scarce during last 20 years (Valkiūnas and Iezhova, 2004; Kim et al., 2009).

Objective and main tasks of the study

The **objective** of this study was to identify some patterns of development of widespread avian malaria parasites (*Plasmodium*) and haemoproteids (*Haemoproteus*) in blood - sucking dipteran insects using experimental approaches.

The following main tasks were set up to achieve this objective:

1. To determine patterns of development of the closely related mitochondrial cytochrome *b* (cyt *b*) gene lineages pSGS1 and pGRW11 of *Plasmodium relictum* and different isolates of the lineage pSGS1 in the blood sucking mosquitoes *Culex pipiens pipiens* (Culicidae).

2. To determine if the lineages pSGS1 and pGRW11 of *Plasmodium relictum* complete sporogony in mosquitoes *Culex pipiens pipiens* form *molestus.*

3. To determine the vectors of *Haemoproteus minutus* and *Haemoproteus belopolskyi* (Haemoproteidae).

4. To determine peculiarities of the development of *Haemoproteus* parasites in blood - sucking mosquitoes (Culicidae).

5. To determine virulence of *Haemoproteus* parasites for bird - biting mosquitoes and the mechanism of the virulence in these insects.

6. To determine development of *Plasmodium homocircumflexum* (lineage COLL4) in mosquitoes *Culex pipiens pipiens, Culex pipiens pipiens* form *molestus* and *Aedes vexans.*

Statements to be defended:

1. Sporogony of the lineages pSGS1 and pGRW11 of *Plasmodium relictum* and different isolates of the lineage pSGS1 completes in the mosquitoes *Culex pipiens pipiens*.

2. The lineages SGS1 and GRW11 of *Plasmodium relictum* produce morphologically indistinguishable sporogonic stages and develop synchronously to the sporozoite stage in the mosquitoes *Culex pipiens pipiens* form *molestus.*

3. The biting midge *Culicoides impunctatus* (Ceratopogonidae) is an important vector of avian *Haemoproteus* species, including *Haemoproteus minutus* and *H. belopolskyi*.

4. *Haemoproteus* parasites can develop to the oocyst stage in mosquitoes.

5. Determination of haemosporidian vectors based solely on polymerase chain reaction (PCR) - based methods is unreliable. Combination of microscopy and PCR - based methods is essential in haemosporidian vector research, particularly in wildlife.

6. The migrating *Haemoproteus* spp. ookinetes damage organs throughout the entire body of infected mosquitoes; that causes high mortality of the heavily infected insects.

7. The sporogonic development of *Plasmodium homocircumflexum* (lineage pCOLL4) occurs to the oocyst stage in three common European mosquitos, i. e. *Culex pipiens pipiens, Culex pipiens pipiens* form *molestus* and *Aedes vexans,* but sporozoites do not develop; the sporogony is abortive in all these mosquito species.

Novelty of the study:

1. It was shown for the first time that the lineages pSGS1 and pGRW11 of *Plasmodium relictum* and different isolates of the lineage pSGS1 develop synchronously and produce morphologically indistinguishable sporogonic stages in the mosquito *Culex pipiens pipiens.*

2. This study shows that experimental results show, that *Culex pipiens pipiens* form *molestus* is a convenient experimental vector in avian malaria research.

3. It was proved that *Haemoproteus minutus* and *Haemoproteus belopolskyi* complete sporogony in the biting midge *Culicoides impunctatus* (Ceratopogonidae). Sporogonic stages of these parasites have been described for the first time.

4. It was shown for the first time that the sexual processes and the development of ookinetes of *Haemoproteus* parasites occur throughout the entire digestive tract of the engorged mosquitoes *Ochlerotatus cantans*, including the head, thorax and abdomen. The sporogonic development is abortive in mosquitoes at oocyst stage. The currently used PCR - based diagnostics detects these parasites in the experimentally infected insects up to 17 day post infection (dpi), but does not distinguish the abortive haemosporid ian development.

5. *Haemoproteus* ookinetes find no barrier in their way from the gut contents to the gut wall of the infected mosquitoes *Ochlerotatus cantans*, resulting in the presence of the parasites throughout the body of the infected insects and their high mortality.

6. It was shown for the first time that the sporogonic development of *Plasmodium homocircumflexum* (lineage pCOLL4) is abortive in the mosquitoes *Culex pipiens pipiens, Culex pipiens pipiens* form *molestus* and *Aedes vexans.*

Scientific and practical significance:

1. Determination of susceptible vectors is a difficult task in wildlife research. We suggest using phylogenies based on the mitochondrial cyt *b* gene for predicting vector species of genetically similar avian malaria parasites. That will speed determination of susceptible vectors in wildlife.

2. We recommend *Culex pipiens pipiens* form *molestus* for experimental and epidemiological studies of avian malaria parasites, particularly because of its: a) widespread distribution; b) high susceptibility to different lineages of *Plasmodium relictum,* and c) easy to establish colonies of wild - collected insects for experimental research at laboratory conditions.

3. The biting midge *Culicoides impunctatus* is involved in the transmission of deadly *Haemoproteus minutus*, which kills birds in Europe. This biting midge worth more attention in research of avian haemoproteosis.

4. Solely PCR - based diagnostics should be carefully used in vector studies of haemosporidians because it detects parasites in insects for several weeks after initial infection, but does not distinguish abortive parasite development. Dem-

onstration of infective sporozoites in insects is essential for definitively demonstrating that certain insect species are vectors. A combination of PCR - based and microscopic methods is a gold standard in haemosporidian vector research, particularly in wildlife.

5. New knowledge about high virulence of *Haemoproteus* parasites to mosquitoes worth attention from the point of view of transmission of haemosporidian parasites and, probably other mosquito - born infections. Markedly reduced survival of heavily infected blood - sucking insects due to haemoproteosis implies a possible reduced involvement in transmission of other infections by decreasing the number of survived biting females and the egg production in mosquito population. We point out that this might be significant epidemiologically.

6. We recommend using haemosporidian parasite molecular makers in determining possible links between blood - sucking insects and their blood - source animals. That will help to determine feeding preference of blood - sucking insects in wildlife.

Approbation of results. The results of this study have been published in 9 publications. Among them 6 full articles in international journals listed in the Web of Science database and 3 abstracts of scientific conferences. Results of the dissertation were presented at the international workshop "Disease vectors today: Changes in ecology, climate and public health risks" (Saaremaa, Estonia, 2011, May 24 – 27th), at the "5th Conference of the Scandinavian - Baltic Society for Parasitology" (Copenhagen, Denmark, 2013, September 10 – 13th) , and at the "International conference on malaria and related haemosporidian parasites of wildlife" (Vilnius, Lithuania, 2013, August 7 – 11th). This dissertation work has been discussed and approved at annual meetings of PhD students at Nature Research Centre, Vilnius in 2011, 2012, and 2013.

Structure of the study. The dissertation is presented in the following chapters: *Introduction*, *Literature review*, *Material and Methods*, *Results and Discussion*, *Conclusions*, *Acknowledgements*, *References*, and *the List of the Author's Publications*. References include 143 sources. The dissertation contains 90 pages, 4 tables and 13 figures. The text of the dissertation is in English, with a summary in Lithuanian.

1. LITERATURE REVIEW

1.1. Brief description of the life cycle of *Haemoproteus* and *Plasmodium* (Haemosporida) parasites in blood - sucking dipteran insects

General scheme of life cycles of haemosporidian parasites is well - known (Garnham, 1966; Valkiūnas, 2005), but complete life cycles and details of development of the majority of species of bird *Haemoproteus* and *Plasmodium* parasites remain non - studied or have been investigated fragmentarily (Bishop and Bennett, 1992; Valkiūnas, 1997; 2008b). During haemosporidian development the parasites change hosts, i. e. the vertebrates (birds) and vectors (blood - sucking dipterans, Insecta: Diptera). Life cycles of bird *Haemoproteus* (Fig. 1) and

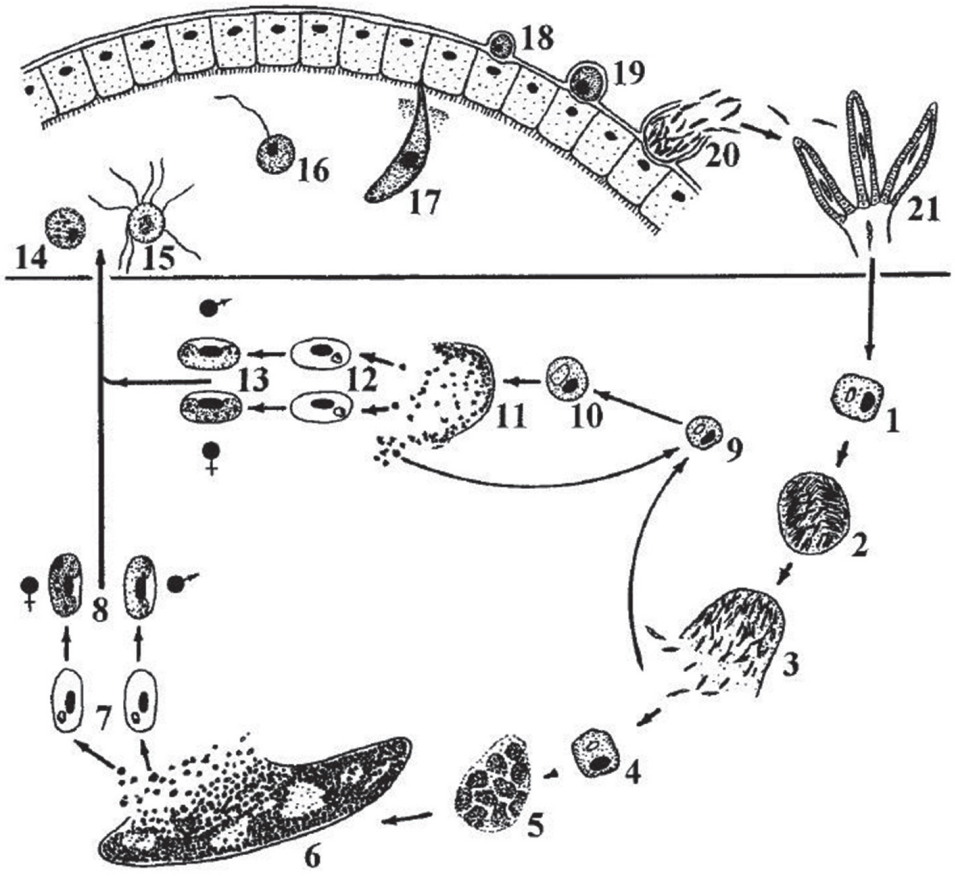

Fig. 1. Diagrammatic representation of the life cycle of bird haemoproteids (*Haemoproteus mansoni* as an example).
Upper part, in vector; lower part, in bird: *1* – sporozoite in endothelial cell; *2*, *3* – exoerythrocytic meronts of the first generation with elongated merozoites; *4* – merozoite in endothelial cell; *5*, *6* – growing and mature megalomeronts in skeletal muscles, respectively; *7* – merozoites in erythrocytes; *8* – mature gametocytes; *9* – merozoite in reticuloendothelial cell in spleen; *10*, *11* – growing and mature meronts in spleen, respectively; *12* – merozoites in erythrocytes; *13* – mature gametocytes; *14* – macrogamete; *15* – exflagellation of microgametes; *16* – fertilization of macrogamete; *17* – ookinete penetrating the peritrophic membrane; *18* – young oocyst; *19*, *20* – sporogony; *21* – sporozoites in the salivary glands of vector (according to Valkiūnas, 2005).

Plasmodium (Fig. 2) parasites are similar, but have some significant differences. Biting midges (Diptera: Ceratopogonidae) and louse flies (Hippoboscidae) are the vectors of bird haemoproteids (Atkinson et al., 1986). Blood - sucking mosquitoes (Diptera: Culicidae) are vectors of malaria parasites of birds (Valkiūnas, 2005; LaPointe et al., 2010; Santiago Alarcon, 2012).

Species of the genera *Culex*, *Aedes*, *Culiseta* and *Anopheles* and some others are involved in the transmission of *Plasmodium* parasites (Garnham, 1966). Biting midges of the *Culicoides* (Ceratopogonidae) and louse flies of the Hippoboscidae transmit different species of the Haemoproteidae. According to the

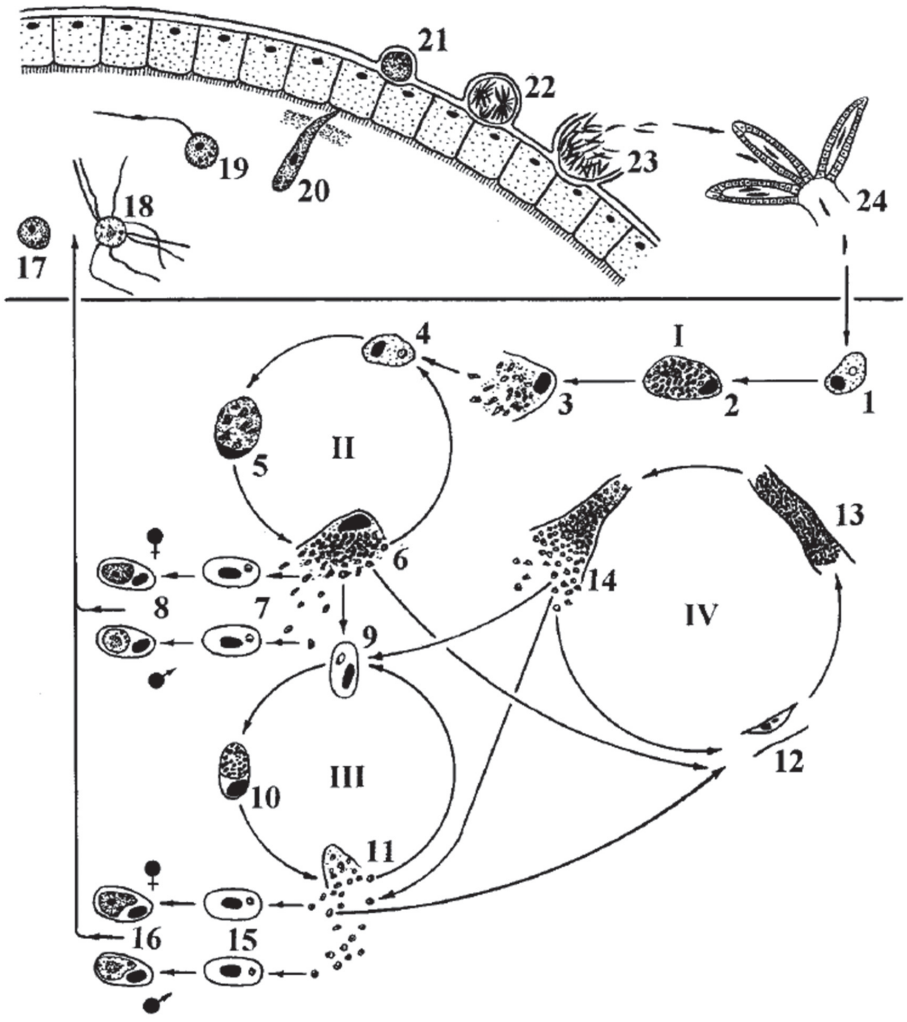

Fig. 2. Diagrammatic representation of the life cycle of bird malaria parasites (*Plasmodium relictum* as an example):
Upper part, in vector; lower part, in bird: I, II – primary exoerythrocytic merogony; III – erythrocytic merogony; IV – secondary exoerythrocytic merogony; *1* – sporozoite in reticuloendothelial cell; *2*, *3* – cryptozoites; *4* – merozoite in macrophage; *5*, *6* – metacryptozoites; *7* – merozoites in erythrocytes; *8* – gametocytes; *9* – merozoite in erythrocyte; *10*, *11* – erythrocytic meronts; *12* – merozoite in endothelial cell of capillaries; *13*, *14* – phanerozoites; *15* – merozoites in erythrocytes; *16* – gametocytes; *17* – macrogamete; *18* – exflagellation of microgametes; *19* – fertilization of macrogamete; *20* – ookinete penetrating the peritrophic membrane; *21* – young oocyst; *22*, *23* – sporogony; *24* – sporozoites in the salivary glands of vector (according to Valkiūnas, 2005).

current knowledge, the general chime of the life cycle of avian haemosporidian parasites is as follows (Valkiūnas, 2005; Atkinson, 2008). Sporogony occurs in the midgut, and sporozoites penetrate into the salivary glands of the blood - sucking insects, which become capable of infecting the birds during their next blood meal (Figs. 1, 2). The sexual process takes place in the vectors, and thus the birds are intermediate hosts and the vectors are final (definitive) ones. While feeding, the vectors inoculate sporozoites in birds giving rise to agamic stages, which undergo asexual division in the cells of the fixed tissues of the host (Figs. 1, 2). These stages are known as exoerythrocytic meronts or schizonts. As a result of multiple or agamous division (merogony or schizogony) in meronts, uninuclear merozoites are formed which are asexual stages of spreading within the organism of the host. Usually, there are several generations of the exoerythrocytic development, during which the parasite gradually adjusts to the host (Figs. 1, 2). Blood stages (erythrocytic meronts and gametocytes) develop in red blood cells. *Haemoproteus* and *Plasmodium* parasites produce malarial pigment (hemozoin) in the cytoplasm of the red blood cells.

After feeding on infected birds, the gametocytes initiate gametogenesis, which usually occurs in the midgut of the vectors, resulting in a sexual process of the oogamy type (Figs. 1, 2). A macrogametocyte produces one rounded macrogamete, while a microgametocyte undergoes exflagellation, as a result of which eight motile thread-like microgametes are formed. Fertilization occurs extracellularly (Figs. 1, 2). The zygote is transformed into ookinete. The latter penetrates through a peritrophic membrane and through the epithelial layer of the midgut. The ookinete rounds up under the basal lamina and develops into an oocyst, which is surrounded by a capsule - like wall built from the material of the host (Figs. 1, 2). After maturation of the oocysts, the sporozoites move into the haemocoel and then penetrate the salivary glands of the vector. Sporozoites are infective to birds.

The live cycles of *Haemoproteus* and *Plasmodium* parasites are similar (Figs. 1, 2), but have some important major differences, the most distinctive of which are the following. First, meronts of haemoproteids (Fig. 1) develop only in the internal organs (only exoerythrocytic merogony is available), but meronts of *Plasmodium* parasites (Fig. 2) develop both in the organs and in erythrocytes (both exoerythrocytic and erythrocytic merogony is available) (Atkinson, 2008; Valkiūnas, 2005). This feature is very handy in identifying genera of these parasites (Garnham, 1966). Second, tissue meronts of some *Plasmodium* species can

develop in stem haemopoietic cells, which is not a case in *Haemoproteus* spp. (Atkinson, 2008). Third, only large oocyst (diameter > 20 μm, Fig. 2) develop in species of *Plasmodium* (Garnham, 1966; Valkiūnas, 2005). Such large oocysts have been described only in several species of haemoproteids of the subgenus *Haemoproteus*, but absent from species of the subgenus *Parahaemoproteus* (Fig. 1, Valkiūnas, 2005). However details of sporogonic development of the majority of described species of avian malaria parasites and haemoproteids remain insufficiently studied and comparative studies are few (Garnham, 1966; Atkinson, 2008; 1991a).

Because complete life cycles of many species of avian haemosporidians, particularly during sporogony, remain insufficiently investigated (Valkiūnas, 2002; 2013c; Ejiri et al., 2009; Njabo et al., 2011; Levin, 2013), we designed several experiments aiming better understanding peculiarities of development of these parasites in vectors.

1.2. Review of studies of *Haemoproteus* and *Plasmodium* parasites in blood - sucking insects related to the dissertation

1.2.1. Brief historical review on determining vectors of avian Haemoproteus and Plasmodium parasites

First description of the development of the malaria parasite (*Plasmodium relictum*) in unidentified to species mosquito (genus *Culex*) and the confirmation of the transmissive way of spreading malaria was made by Ross (1898). This discovery is often considered as the peak achievement of malariology in the 19th century. Ronald Ross was awarded the Nobel Prize in Medicine for this discovery in 1902. Since this discovery, bird malaria parasites have began to attract the attention of scholars as an experimental model for the investigation of human malaria, and they were used for this purpose in many laboratories of that time. Koch (1899) was one of the first to confirm the results obtained by R. Ross infecting domestic canaries with malaria by means of mosquito bites.

Since R. Ross discovered the vectors of *Plasmodium* spp., about 60 years were needed to determine the range of invertebrate hosts of other bird haemosporidians (Garnham, 1966). Sergent brothers (Sergent and Sergant, 1906) established that the hippoboscid fly *Lynchia maura* (syn. *Pseudolynchia canariensis*) is able to transmit *Haemoproteus columbae* from one domestic pigeon to another. Soon after this, the sporogony of this parasite was studied and described in detail by Adie (1915).

After discovery of avian malaria transmission and high susceptibility of domestic canaries to *Plasmodium relictum*, this bird became the most attractive experimental hosts as a donor of gametocytes to infect vectors in malaria research at many laboratories for many decades (Valkiūnas, 2005), and it retained this status up to the discovery of the domestic chicken parasite *Plasmodium gallinaceum* in the second half of the 1930s. We used the canary as donors of gametocytes in this dissertation study.

Bennett et al. (1965) established two new genera (*Parahaemoproteus* and *Akiba*) for the species of haemosporidians whose vectors are biting midges (*Akiba* belongs to the Leucocytozoidae). Haemoproteids transmitted by louse flies (Hippoboscidae) were placed to the genus *Haemoproteus*, and those transmitted by ceratopogonid midges were placed to the *Parahaemoproteus*. Both these genera are considered as subgenera of the genus *Haemoproteus* in the majority of recent classifications (Valkiūnas, 2005; Perkins, 2014).

Due to application of polymerase chain reaction PCR - based methods, the field of molecular biology of avian hemosporidians has been rapidly developing (Marzal et al., 2005; Martinsen et al., 2008; Bensch et al., 2009; Ricklefs and Outlaw, 2010). Many current molecular studies address occurrence of wildlife hemosporidian parasites in avian hosts and vectors (Palinauskas et al., 2008; Zehtindjiev et al., 2008; Vézilier et al., 2010; Valkiūnas et al., 2014b). PCR - based methods provide straightforward opportunities for investigation of feeding specialisation of blood - sucking insects and other arthropods; it is easy to detect and identify DNA of blood source animals in engorged insects and thus to predict links between vectors and their hosts (Malmqvist et al., 2004; Ejiri et al., 2011; Kim and Tsuda, 2012). However, the opportunities to use the host DNA markers are limited in such research because of rapid DNA degeneration in the digestive tract of insects (Kim and Tsuda, 2012).

PCR - based detection methods accelerate search of most probable vector species by determining significant links between blood - sucking insects, on the one hand and haemosporidian species, on the other hand (Ejiri et al., 2011). However, it remains unclear if all PCR - positive signals can be used in determining vectors of the parasites due to possible abortive haemosporidian infections. We predict that the report of sporozoites in blood - sucking insect fed on infected hosts is an essential for the definitive demonstration that blood sucking insects could act as vectors. Solely the PCR - based diagnostics is likely insufficient in determinations of vectors. One of the experiments of this dissertation was designed to investigate this issue.

1.2.2. Investigation of sporogony of the Plasmodium relictum genetic lineages in mosquitoes Culex pipiens pipiens

Recent PCR - based studies provided innovative opportunities to diagnose malaria parasites and revealed huge genetic diversity of these pathogens, which are convenient model organisms to address questions about evolutionary biology of malaria and other pathogens (Perkins and Shall, 2002; Bensch et al., 2004; Ricklefs et al., 2004; Møller and Nielsen, 2007). There is several fold increase in both citations and publications on avian malaria over the past decade (Bensch et al., 2009). That indicates the rapid increase of interest on avian *Plasmodium* spp. and related haemosporidians in zoology (Iezhova et al., 2011; Zehtindjiev et al., 2012), veterinary and conservation medicine (Levin et al., 2009; Santiago-Alarcon et al., 2010; Olias et al., 2011), and particularly in evolutionary biology (Perez-Tris and Bensch, 2005; Møller and Nielsen, 2007; Bensch et al., 2009; Santiago-Alarcon et al., 2010; Ricklefs and Outlaw, 2010; Levin et al., 2011; Marzal et al., 2011; Loiseau et al., 2012).

Numerous PCR - based and morphological studies addressed taxonomy, distribution, evolutionary biology and other issues of avian *Plasmodium* spp. biology, resulting in deposition of numerous parasite lineages in GenBank (Beadell et al., 2006; Dimitrov et al., 2010; Knowles et al., 2010; Marzal et al., 2011; Szollosi et al., 2011; Loiseau et al., 2012). However, information about development of different *Plasmodium* lineages in vectors remains insufficient (Ejiri et al., 2009; Kim et al., 2009; Kimura et al., 2010; LaPointe et al., 2010; Njabo et al., 2011). Comparative studies on sporogonic development of closely related lineages and different isolates of the same lineage in mosquito vectors are lacking. That is an obstacle to address epidemiology questions related to avian malaria transmission and also bias the rapidly accumulating information on *Plasmodium* spp. lineage diversity.

P. relictum lineages pSGS1 and pGRW11 have been reported in over 30 species of birds in the Old World, but both lineages are rare in the New World (Palinauskas et al., 2007; Bensch et al., 2009; Marzal et al., 2011). However, remarkably little is known about sporogony of these parasite lineages (Vezilier et al., 2010). One of the tasks this study was to compare sporogonic development and morphology of 2 widespread lineages of *P. relictum* (pSGS1 and pGRW11) in mosquito *C. p. pipiens* (Linnaeus, 1758).

1.2.3. Investigation of sporogony of the Plasmodium relictum genetic lineages in mosquitoes Culex pipiens pipiens form molestus

Culex p. pipiens f. *molestus* (Forskal, 1775) is widespread in the Holarctic. It is characterized by broad ecological plasticity (Gomes et al., 2009; Vinogradova, 2000) and is particularly common in human settlements where it often breeds in sewers, but also occurs in natural sheltered habitats such as caves and other similar ecological niches. Both *C. p. pipiens* and *C. p. pipiens* f. *molestus* often occur sympatrically, particularly in countries with mild winters (Osorio et al., 2014). *Culex p. pipiens* is an important vector of avian malaria (Santiago-Alarcon et al., 2012). However, the role of *C. p. pipiens* f. *molestus* in transmission of avian malaria remains unclear. Because of peculiarities of swarming and mating behavior of *C. p. pipiens*, it is difficult to establish its new colonies and to maintain them under laboratory conditions using wild - collected insects (Vinogradova et al., 1996). That makes obstacles to use this mosquito in the experimental research aiming better understanding geographical variation in susceptibility of vectors to avian malaria. *C. p. pipiens* f. *molestus* differs from *C. p. pipiens* by: 1) autogeny of females, 2) stenogamy, 3) high degree of anthropophily, and 4) absence of overwintering in diapauses (Vinogradova et al., 1996; Becker et al., 2003). These features allow *C. p. pipiens* f. *molestus* to be easily cultivated in small mosquito cages under laboratory conditions using wild - sampled insects, making this species convenient model for experimental studies. However, it remains unclear if *C. p. pipiens* f. *molestus* worth attention in epidemiology of avian malaria. Only one study reported complete sporogonic development of one species of malaria parasite, i. e., *Plasmodium (Giovannolaia) garnhami* in *C. p. pipiens* f. *molestus* (Garnham, 1966). Because this mosquito form has not been reported in the list of vectors of the great majority of avian malaria parasites (Valkiūnas, 2005; Santiago-Alarcon et al., 2012), the task of study was to investigate if two widespread *P. relictum* lineages (pSGS1 and pGRW11) complete sporogony in this insect.

1.2.4. Haemoproteus spp. sporogony in the biting midges

Numerous recent studies addressed molecular characterization, distribution and genetic diversity of haemoproteids. However, few studies deal with vectors and transmission of avian *Haemoproteus* spp. (Atkinson, 1991a; Desser and Bennett, 1993; Valkiūnas et al., 2002; Martínez-de la Puente et al., 2011; Santiago-Alarcon et al., 2012; Levin et al., 2013). Biting midges of *Culicoides* (Diptera,

Ceratopogonidae) and louse flies (Hippoboscidae) can transmit these parasites, but certain vector species remain unknown for the great majority of avian haemoproteids and their lineages (Atkinson, 2008; Clark et al., 2014).

The sporogony of only few *Haemoproteus* spp. has been investigated in detail in biting midges (Linley, 1985; Valkiūnas, 2005; Santiago-Alarcon et al., 2012); it is difficult to work with these insects due to their tiny size and difficulties to colonize the majority of their species (Miltgen et al., 1981; Valkiūnas, 2005; Atkinson, 2008). It was shown that *Culicoides impunctatus* (Goetghebuer, 1920) transmits several species of haemoproteids in Europe (Valkiūnas, 2005). This biting midge was reported as a vector of *Haemoproteus belopolskyi* from blackcaps *Sylvia atricapilla* (Valkiūnas and Iezhova, 2004). However, recent molecular studies show that this blackcap parasite is actually *Haemoproteus parabelopolskyi* (Valkiūnas et al., 2007), and the vector of *H. belopolskyi*, which parasitize icterine warblers *Hippolais icterina* needs to be identified.

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

1.2.5. Development of Haemoproteus parasites in mosquitoes

Approximately 150 species of avian haemoproteids have been described (Iezhova et al., 2011). The majority of these belong to the subgenus *Parahaemoproteus* and are transmitted by biting midges belonging to *Culicoides* (Ceratopogonidae). Currently, only 10 species have been assigned to the subgenus *Haemoproteus*, all of which are transmitted by hippoboscid flies of the Hippoboscidae (Bennett et al. 1965; Garnham 1966; Valkiūnas et al., 2010; Levin et al., 2012; Valkiūnas et al., 2013a). According to current knowledge, only biting midges and hippoboscid flies act as vectors of avian haemoproteids (Baker, 1966; Garnham, 1966; Atkinson and van Riper, 1991a; Desser and Bennett, 1993; Valkiūnas, 2005). That is in accord with the molecular phylogeny studies suggesting that evolution of *H.* (*Parahaemoproteus*) and *H.* (*Haemoproteus*) parasites is closely associated with adaptation to species of the Ceratopogonidae and Hippoboscidae, respectively (Martinsen et al., 2008; Santiago-Alarcon et al., 2010; Levin et

al., 2012). However, vector species have only been identified with certainty for very few *Haemoproteus* species (Valkiūnas, 2005; Santiago-Alarcon et al., 2012). Such studies are difficult to design using traditional parasitology methods, particularly in wildlife because haemoproteids do not multiply in the circulation, so parasite strains cannot be maintained in birds by means of infected blood inoculation, which is easy to do in the case of avian malaria. Experimental infection of birds with haemoproteids needs injection of infective sporozoites into susceptible avian hosts. In practice, determination of susceptible vectors of these parasites requires experimental infection of blood - sucking insects by feeding them on naturally infected birds, many species of which are difficult to maintain in captivity (Baker, 1957; Fallis and Bennett, 1960; Atkinson and van Riper, 1991b; Desser and Bennett, 1993; Valkiūnas et al., 2002). Such experiments are particularly difficult to do with haemoproteids parasitising rare bird species and in remote areas.

Molecular markers provide new opportunities to detect haemosporidian lineages during light infections both in avian hosts and vectors. Mitochondrial cyt *b* gene markers have been successfully applied for detecting and distinguishing haemosporidian species and are easy to use (Martinsen et al., 2006; Sehgal et al., 2006; Palinauskas et al., 2007; Santiago-Alarcon et al., 2010; Iezhova et al., 2011; Martínez-de la Puente et al., 2011; Križanauskienė et al., 2012). It is attractive to apply the PCR - based methods in vector research (Ejiri et al., 2009; Njabo et al., 2009; Kimura et al., 2010; Ejiri et al., 2011; Martínez-de la Puente et al., 2011; Foley et al., 2012; Glaizot et al., 2012; Kim and Tsuda, 2012; Ventim et al., 2012; Kazlauskienė et al., 2013). Several recent studies reported the presence of *Haemoproteus* spp. lineages in mosquitoes and speculated about possible involvement of these insects in transmission (Ishtiaq et al., 2008; Njabo et al., 2011). However, sporogonic development of *Haemoproteus* spp. lineages has not been documented in mosquitoes.

One of the tasks of this study was to study experimentally development of *Haemoproteus* (*Parahaemoproteus*) *tartakovskyi* and *Haemoproteus* (*Parahaemoproteus*) *balmorali* in *Ochlerotatus cantans* (Meigen, 1818) (Diptera, Culicidae), a widespread Eurasian mosquito. These haemosporidian parasites are common and prevalent in siskin *Carduelis spinus* and thrush nightingale *Luscinia luscinia* in Europe, respectively. We aimed to investigate sporogonic development and survival time of these haemoproteids in mosquito *O. cantans* using microscopic and PCR - based examination methods, which were applied

in parallel. This mosquito is a member of the tribe Aedini, many species of which transmit closely related avian *Plasmodium* parasites (Valkiūnas, 2005; Santiago-Alarcon et al., 2012).

1.2.6. The virulence of Haemoproteus infections to blood - sucking insects

Some haemoproteids cause severe diseases in avian hosts and affect their fitness (Atkinson et al., 1988; Merino et al., 2000; Marzal et al., 2005; Cannell et al., 2013). However, little is known about the effects of *Haemoproteus* infections on blood - sucking insects Valkiūnas and Iezhova (2004) reported mortality of the biting midge *Culicoides impunctatus* (Diptera, Ceratopogonidae) associated with experimental infections of *Haemoproteus belopolskyi*, *H. fringillae* and *H. lanii*. This biting midge transmits several species of *Haemoproteus* in Europe (Valkiūnas, 2005). However, effects of haemoproteids on mosquitoes and other bird - biting insects are insufficiently investigated.

Mosquitoes do not transmit *Haemoproteus* parasites and should be not adapted to this infection (Garnham, 1966; Ejiri et al., 2009; Kim et al., 2009; Santiago-Alarcon et al., 2012). A recent experimental study (Valkiūnas et al., 2013c) showed that several species of *Haemoproteus* develop numerous ookinetes and early oocysts throughout the entire body of mosquitoes *Ochlerotatus cantans*; these parasites do not complete sporogony, which is abortive at the oocyst stage. It was speculated that *Haemoproteus* infections might be virulent to bird - biting mosquitoes. To our knowledge, there are no other records about effects of haemoproteids on longevity of blood - sucking mosquitoes. However, these insects have been reported to be PCR positive for *Haemoproteus* spp. lineages across the world (Ishtiaq et al., 2008; Njabo et al., 2011; Glaizot et al., 2012; Synek et al., 2013; Valkiūnas et al., 2013c). Experimental observations are needed for a better understanding of the damage caused by *Haemoproteus* parasites in mosquitoes and other bird - biting insects. Because such studies are uncommon, one of our tasks was to follow the survival rate of the mosquito *O. cantans* following experimental infection with three widespread species of avian haemoproteids, i.e. *H.* (*Parahaemoproteus*) *balmorali*, *H.* (*Parahaemoproteus*) *lanii* and *H.* (*Parahaemoproteus*) *tartakovskyi*. This mosquito is widespread in Eurasia and readily bites birds (Bernotienė, 2012). We allowed wild - caught females to feed on naturally infected and uninfected birds and followed fate of the exposed insects at controlled laboratory conditions.

1.2.7. Sporogonic development of Plasmodium homocircumflexum (lineage COLL4) in mosquitoes

Analysis of cyt *b* gene sequences based on PCR revealed huge diversity of lineages of avian haemosporidian parasites (Ishtiaq et al., 2007; Beadell et al., 2009; Bensch et al., 2009; Chasar et al., 2009; Dimitrov et al., 2010; Marzal et al., 2011; Levin et al., 2013). However sporogonic development of the majority of species and their lineages of avian *Plasmodium* spp. remains unknown (Kim et al., 2009; LaPointe et al., 2010; Njabo et al, 2011; Palinauskas et al., 2007). We have discussed this issue in paragraph 1.2.2 in more detail.

Recently, we isolated new malaria parasite, *Plasmodiun homocircumflexum* (pCOLL4) from a naturally infected red - backed shrike *Lanius collurio*. Because sporogony of this parasite in unknown, the task of this study was to provide information about sporogonic development of this parasite in some common European mosquito species, i. e. *Culex pipiens pipiens*, *Culex pipiens pipiens* form *molestus* and *Aedes vexans* (Meigen, 1830).

2. MATERIALS AND METHODS

Each experiment of this dissertation has numerous precise methodological differences and certain experimental design. To facilitate repeatability of this study, description of the materials and methods is given for each experiment separately.

Experimental procedures of this dissertation were approved by the Lithuanian State Food and Veterinary Office (protocol no. 2012/01/04 – 0221) and were in accordanve to the International Research Co-operation Agreement between the Biological Station Rybachy of the Zoological Institute of the Russian Academy of Sciences and Institute of Ecology of Nature Research Centre (25 – 05 – 2010). All efforts were made to minimize handling time and potential suffering of animals. None of the experimental birds suffered apparent injury during experiments.

2.1. Study site

This work was carried out between 2010 and 2014. The field studies and part of experimental work were carried out at the Biological Station of the Zoological Institute of Russian Academy of Sciences on the Curonian Spit in the Baltic Sea between May and August each yaer. This study site locates in the village Rybachy (Fig. 3). Near this village, the main forest creating species are broad - leaved woods and pines (*Pinus sylvestris*, *Picea abies*, also *Betula pendula*, *Betula pubescens*, *Alnus glutinosa*, *Alnus incana*, *Populus tremula* and other).

The experiments with canary parasites were done at the P. B. Šivickis Laboratory of Parasitology, Nature Research Centre, Vilnius.

Fig 3. Study site. Location of the Biological Station "Rybachy".

2.2. Experiment 1: Determination of sporogony of *Plasmodium relictum* (lineages pSGS1 and pGRW11) in mosquitoes *Culex pipiens pipiens*

2.2.1. The lineages of Plasmodium relictum

We used three *P. relictum* lineages, which were isolated from naturally infected house sparrow *Passer domesticus* (lineage pGRW11, Gen – Bank accession number JX993047), common crossbill *Loxia curvirostra* (pSGS1, JX993045) and domestic canary *Serinus canaria domestica* (pSGS1, JX993046). Strains of the lineage pGRW11 and the crossbill lineage pSGS1 were obtained from the bank of cryopreserved avian malaria parasites, which is available in the P. B. Šivickis Laboratory of Parasitology, Nature Research Centre, Vilnius, Lithuania. Before cryopreservation, these strains were multiplied in siskins *Carduelis spinus* according to Palinauskas et al. (2008). At the time of the experiments, both these strains had undergone 2 passages since their original isolation from the wild birds. The canary pSGS1 strain was isolated from a naturally infected domestic canary imported to Lithuania from Slovak Republic in 2011. Parasitemia was light (< 0.0001 %) in this bird. To multiply this strain, 2 experimental canaries were infected by intramuscular inoculation of approximately 50 μl of blood from the infected canary, as described by Palinauskas et al. (2008). The first passage of this strain was used in the present study.

2.2.2. Experimental canaries and maintenance of Plasmodium relictum in birds

Fourteen domestic canaries were commercially purchased and used for experiments and controls. After arrival to the laboratory, the birds were kept at quarantine in mosquito free room for one month. The birds' blood was tested for malaria parasites on the days 15 and 30 after their arrival to the laboratory. All birds were uninfected with malaria parasites as revealed by microscopic examination of blood films and PCR - based detection method (see description of these methods below). To multiply *P. relictum* strains for mosquito experiments, 9 canaries were infected by inoculation of infected blood in pectoral muscle of birds, as described by Palinauskas et al. (2008). Three birds were infected with each parasite strain. Parasitemia developed in all infected canaries; these birds were used as donors of gametocytes to infect mosquitoes. Five uninfected canaries were used as negative controls. To monitor parasitemia, blood for microscopic examination and PCR - based analysis was taken from the 3rd dpi each 2rd or 3rd day onwards. Control birds were tested at the same days. We also

evaluated parasitemia in all donor canaries immediately after mosquito blood meal on birds.

2.2.3. Collection of blood samples and their microscopic and PCR - based examination

The blood was taken from birds by puncturing the brachial vein. About 50 μl of blood was taken in heparinized microcapillaries and stored in SET - buffer (Hellgren et al., 2004) for molecular analysis.

Two blood films were prepared from each bird immediately after withdrawal. They were air - dried, fixed in absolute methanol and stained with Giemsa, as described by Valkiūnas et al. (2008a). Approximately 100 – 150 fields were examined at low magnification (× 400), and then at least 100 fields were studied at high magnification (× 1000). 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. (1987). Malaria parasites were identified according to Valkiūnas (2005) and Palinauskas et al. (2007). All canaries were examined for malaria parasites by PCR - based detection method.

The total DNA from blood was extracted using innuPREP Blood DNA Mini Kit (Analytikjena, Berlin, Germany). For genetic analysis, we used a nested PCR protocol amplifying a segment of 478 nucleotides of parasite mitochondrial cyt *b* (Bensch et al., 2000; Hellgren et al., 2004). In the first PCR, general for haemosporidian parasites primers HaemNFI and HaemNR3 were used (Hellgren et al., 2004). In the second PCR, the primers specific to *Haemoproteus* and *Plasmodium* spp. HAEMF and HAEMR2 were used (Bensch et al., 2000). The amplification was evaluated by running 1.5 μl of the final PCR product on a 2 % agarose gel. One negative control (nuclease - free water) and 1 positive control (*Plasmodium* spp. microscopy positive blood sample) were used per every 8 samples to control for false amplifications. No cases of false positive samples were found. Fragments of DNA from the positive samples were sequenced from the 5′ end with the primer HAEMF in the Institute of Biotechnology, Vilnius University. The "Basic Local Alignment Search Tool" (National Centre for Biotechnology Information website: <http://www.ncbi.nlm.nih.gov/BLAST>) was used to determine the lineage of detected parasite sequences. Detected sequences were deposited in GenBank (accessions numbers JX993045 – JX993047).

2.2.4. Phylogenetic analysis

To compose a data set for Bayesian analysis, we used 18 mitochondrial cyt *b* sequences (478 bp fragments). Bayesian phylogeny was constructed using mrBayes version 3.1.2 (Ronquist and Huelsenbeck, 2003). We used the General Time Reversible Model including rate variation among sites (GTR+I+G), as an appropriate model of sequence evolution suggested by the mrModeltest 2v software (Nylander, 2004). The analysis was run for a total of 10 million generations with a sample frequency of every 100 generations. Before constructing a majority consensus tree, 25 % of the initial trees in each run were discarded as "burn in" periods. The remaining trees were used to construct a Majority rule consensus tree. Bootstrap support for branches was estimated based on 1000 replicates. Phylogeny was visualized using Tree View 1.6.6; the software is available from website: <http://evolution.genetics.washington.edu/phylip/software.html>. The sequence divergence between lineages was calculated using a Jukes - Cantor model of substitution, with all substitutions weighted equally, implemented in the program MEGA 4 (Tamura et al., 2007; Kumar et al., 2004).

2.2.5. Collection, maintainance and identification of mosquitoes

Hibernating mosquitoes were collected from 2 basements in Vilnius, Lithuania in January 2012. They were kept in mosquito cages (65 × 65 × 65 cm) in a mosquito room under standard conditions (22 ± 1° C, 60 ± 2 % relative humidity and 14:12 light - dark photoperiod). Several pads of cotton - wool moistened with 10 % saccharose solution were placed in each mosquito cage to feed mosquitoes. After two weeks of laboratory adaptation, the wild-caught mosquitoes were used for experimental infection with parasites. *Culex pipiens* Lineaus and *Culex torrentium* Martini (Martini, 1925) are morphologically similar species existing sympatrically in Lithuania (Pakalniškis et al., 2006). These species can be distinguished by morphology only due to differences of male genitalia (Service, 1968).

To rule out an opportunity of presence of natural malaria infection in wild-caught mosquitoes, we tested 114 haphazardly chosen females of *C. p. pipiens* using PCR - based methods. These insects were collected in the same basements and at the same time as experimental mosquitoes. DNA was extracted from pools of 4 mosquitoes using innuPREP blood DNA Mini kit (Analytic Jena AG). Nested PCR protocol that amplifies a fragment of the cyt *b* gene was used in or-

der to detect malaria parasites, as described above. Two thoraxes of mosquitoes, experimentally infected with *P. relictum*, were used as a positive control during this investigation.

2.2.6 Experimental infection of mosquitoes

Two days before feeding, approximately 30 female mosquitoes were haphazardly chosen from samples collected in different basements and placed inside an experimental cage. Infection of mosquitoes with different lineages of parasites was done in different mosquito cages to prevent mixing parasite lineages. Three such experimental cages were obtained in this way. To increase favor of blood feeding, the mosquitoes in these experimental cages were deprived of saccharose. Canaries, with gametocyte parasitemia of approximately 0.01 % of each strain, were placed in separate mosquito cages. Each infected bird was placed in a plastic tube (length 15 cm, diameter 5 cm) containing a rip, which was used to fix the bird legs. Both tube ends were covered with bolting silk. Only legs were exposed to mosquito bites. Blood feeding was carried out for approximately 1 – 2 h by placing the tubes with birds in mosquito cages. Mosquitoes willingly took blood meal. Each canary was used as a donor to infect mosquitoes once per 4 – 5 days. Experiments with different *Plasmodium* strains were carried out in parallel. Up to 7 mosquitoes were allowed to take blood meal on each bird. Engorged females were taken from the experimental cages with aspirator, placed in separate small insect cages (12 × 12 × 12 cm), kept as described above, and dissected in intervals (see description of dissection methods below). Mosquitoes infected with 3 lineages of parasites were kept and dissected until 32 days post infection (dpi). Ookinetes preparations were prepared on the 1, 2, 3 and 6 dpi; oocysts preparations – on the 6, 8, 10, 12, 14, 16, 20, 24, 28 dpi, and sporozoites preparations – on the same days as oocysts and also on 32 dpi. One or 2 mosquitoes infected with each strain were dissected each day. In total, we infected and dissected 103 mosquitoes; 35 mosquitoes were infected with lineage pGRW11, 38 – with lineage pSGS1 and 30 – with lineage pSGS1 of different isolate, respectively. Thoraxes of dissected mosquitoes were fixed in 96 % ethanol; they were used for PCR analysis (see descriprion of methods above, paragraph 2.2.3) in order to detect malaria infection.

2.2.7. Dissection of mosquitoes and making preparations of ookinetes, oocysts and sporozoites

Infected females of *C. p. pipiens* were lightly anesthetized by putting them into a tube closed with a cotton pad wetted in 96 % ethanol for several minutes. Wings and legs of the insects were removed before dissection, which was performed under the binocular stereoscopic microscope Olympus SZ X 10. Preparations of ookinetes, oocysts and sporozoites were prepared according to Valkiūnas (2005).

Ookinetes locate in the semi - digested contents of the midgut. Content of the midgut was extracted by cutting an extreme segment of the abdomen and pressing the content out on the glass slide. The midgut content was mixed with a minute drop of normal saline and a thin smear was prepared; the smears were dried in the air, fixed with methanol, and stained with Giemza stain in the same way as blood films (see description above).

Permanent preparations of midguts were used to detect oocysts. Midgut was dissected as described by Valkiūnas (2005). A minute drop of 2 % solution of mercurochrome was placed on the freshly dissected midgut, which was then covered with a coverslip and used for preliminary detection of oocysts. Best midgut preparations were used to make permanent oocysts preparations. Midguts oocysts were fixed in 10 % formalin in normal saline for 24 hr. The fixative was replaced with 70 % ethanol. Preparations were washed in distilled water, stained with Erlich's hematoxylin for 10 min, "blued" in tap water containing a pinch of sodium bicarbonate for 5 min, differentiated with acid ethanol until the specimen turns a rusty - reddish color, and then "blued" again for 5 min, dehydrated in 70 % and then 100 % ethanol, cleared by putting a drop of clove oil over the preparation, placed in xylene and, finally mounted in Canada balsam.

Preparations of sporozoites were made after extraction of salivary glands from vectors. Mosquitoes head was cut off and salivary glands were gently pressed out from the thorax; they were placed in a small drop of the normal saline, ruptured by a gentle pressure of a needle, mixed with the normal saline to produce thin small film. The preparations were dried in the air, fixed with absolute methanol, and stained the same way as blood smears.

2.2.8. Microscopic examination of vector preparations and parasite morphology

An Olympus BX – 43 light microscope equipped with Olympus SZX2 – FOF digital camera and imaging software QCapture Pro 6.0, Image - Pro Plius (Tokyo, Japan) was used to examine preparations, prepare illustrations and to take measurements. All preparations were first examined at low magnification (× 200, × 600) and then at high magnification (× 1000). The morphometric features studied (Table 1) were those defined by Valkiūnas (2005). The analyses were carried out using the "Statistica 7" package. Student's t - test for independent samples was used to determine statistical significance between mean linear parameters of parasites. A *P* value of 0.05 or less was considered significant. Voucher specimens of ookinetes (accession numbers 48678 – 48680 NS), oocysts (48681 – 48683 NS) and sporozoites (48684 – 48686 NS) of 3 strains of *P. relictum* were deposited at the Nature Research Centre Vilnius, Lithuania.

2.2.9. Infection of canaries by bites of experimentally infected mosquitoes

To determine infectivity of sporozoites developed in experimentally infected mosquitoes, one female infected with *P. relictum* (pSGS1, 14 dpi) was allowed to take a blood meal on 1 uninfected canary, as described above. After the blood meal, the mosquito was dissected and a preparation of salivary glands was made and examined, as described above. Blood of the canary was taken 3, 5 and 7 dpi; it was tested for malarial infection both by microscopic examination and PCR - based detection, as described above.

2.3. Experiment 2: Determination of sporogony of *Plasmodium relictum* (lineages pSGS1 and pGRW11) in mosquitoes *Culex pipiens pipiens* form *molestus*

2.3.1. The lineages of Plasmodium relictum

We used two lineages of *P. relictum* in this study: 1) the lineage pSGS1 (GenBank accession number JX993045) and 2) the lineage pGRW11 (GenBank JX993047), which were isolated from individual of naturally infected common crossbill *Loxia curvirostra* and 1 individual of house sparrow *Passer domesticus* on the Curonian Spit in the Baltic Sea (55° 09′ N, 20° 52′ E), respectively. These lineages were cryopreserved in liquid nitrogen and stored in the biobank (see paragraph 2.2.1), and these lineages have undergone between 3 and 6 passages

by blood inoculation in crossbills and siskins *Carduelis spinus* since their first isolation.

2.3.2. Experimental canaries and maintenance of Plasmodium relictum in birds

Domestic canaries were used as donors of gametocytes to infect mosquitoes. Other methods of working with birds were the same as described in paragraph 2.2.2.

2.3.3. Collection of blood samples and their microscopic and PGR - based examination has been described in paragraph 2.2.3.

2.3.4. Establishment of mosquitos colony and experimental infection of mosquitoes

To establish a colony of *Culex p. pipiens* f. *molestus*, we used the mosquito larvae, which were obtained from dr. Roland Kuhn. The colony was originally started from larvae collected in Hesse Region (Germany); it has been in continuous culture since 1990. This mosquito laid the first egg batch without blood - feeding, and it readily mated in small cages. Mosquitoes were kept in cages (approximately 65 × 65 × 65 and 120 × 65 × 65 cm) under controlled conditions (relative humidity 60 ± 2%, temperature 22° C ± 1° C, 14:10 hr light/dark (L/D) photoperiod). Several pads of cotton wool moistened in 5 – 10 % saccharose solution and a water bowl for oviposition were placed in each cage.

The parasitemia in all donor canaries was evaluated immediately after mosquito blood meal on donor birds. The intensity of gametocytaemia (percentage of gametocytes per 10 000 red blood cells) in donor canaries infected with the lineages pSGS1 and pGRW11 varied between 0.02 and 0.3 % during exposure of mosquitoes. Mosquitoes were allowed to take blood meals on infected canaries, as described in paragraph 2.2.6. The experimental mosquitoes have never been blood fed.

2.3.5. PCR - based examination

We used 1) the standard ammonium - acetate method to extract genomic DNA from birds' whole blood and 2) innuPREP blood DNA Mini Kit, for extraction of DNA from the thoraxes of mosquitoes. The genetic analysis of parasites was carried out as described in paragraph 2.2.3. This protocol is sensitive in avian malaria diagnostics and has been widely applied in many recent studies (Kazlauskienė et

al., 2013; Perkins, 2014). Method described in the paragraph 2.2.3. The sequences were edited and aligned using BioEdit (Version 7.0.9.0; Hall, 1999). Detected sequences were deposited in the PopSet database of the US National Center for Biotechnology Information; they are available in GenBank (KJ002753 – KJ002755).

2.3.6. Statistical analysis

Statistical analysis were carried out using "Statistica 7" package. Median, the first and third quartiles, and the non-outlier range of oocyst numbers in mosquito midguts were calculated. Mann - Whitney *U* - test was used to compare oocyst numbers recorded in mosquitoes exposed to different lineages of *P. relictum*. Sequence divergence between lineages pSGS1 and pGRW11 was calculated with MEGA version 3.0, using a Jukes - Cantor model of substitution in which all substitutions were weighted equally (Kumar et al., 2004).

2.3.7. Dissection of mosquitoes and making preparations of ookinetes, oocysts and sporozoites

Main methods were descriebd in paragraph 2.2.7. In all, we dissected 40 mosquitoes infected with each parasite lineage in this study. Ookinete preparations were prepared 1 – 2 dpi, oocyst preparations – 10 – 19 dpi, and sporozoite preparations – from 11 until 19 dpi. All oocysts were counted in flattened permanent midgut preparations, which were prepared 12 dpi PCR - based methods were used 1) to determine presence and identity of parasite lineages in birds, 2) to determine parasite lineages in mosquitoes, in which sporozoites were observed. ThePCR - based protocols were the same as described by Kazlauskienė et al. (2013). Representative preparations of blood stages (accession numbers 48764, 48765 NS), ookinetes (48766, 48767 NS), oocysts (48768, 48769 NS) and sporozoites (48770, 48771) were deposited at the Nature Research Centre, Vilnius, Lithuania.

2.4. Experiment 3: Determination of the vectors of *Haemoproteus minutus* and *Haemoproteus belopolskyi* (Haemoproteidae)

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

This study was carried out at the Biological Station in village Rybachy (see paragraph 2.1.) between 21 May and 2 July in 2013.

Birds were captured with mist nets and identified. The blood was collected as described in paragraph 2.2.3. The species of *Haemoproteus* were identified ac-

cording to Valkiūnas (2005). Naturally infected birds with single infections were used as donors to infect biting midges. Blood samples from donor birds were examined for haemosporidian parasites by PCR amplification. Positive amplifications were sequenced and cyt *b* lineages of *Haemoproteus* parasites were determined in the laboratory (see description below).

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

2.4.2. Wild - caught biting midges

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

2.4.3. Experimental design

Wild - caught biting midges were infected as described by Valkiūnas (2005). Briefly, the crown feathers of birds were removed from an area of about 1 cm^2 to facilitate observation of feeding insects, which is helpful during work with such minute flies. Birds were held in hands covered by ruby gloves, and biting midges

were allowed to feed naturally between 21:00 and 23:00 hr on birds at a locality with a high density of flies. *Culicoides impunctatus* willingly takes a blood meal on the feather - free region. The bird's head was inserted into an insect cage when several flies had started to feed. The cages (12 × 12 × 12 cm^3) were made of fine-mesh bolting silk. A zip faster was sewn into one wall of the cage to permit entry of the bird's head and removal of engorged midges, which flew off the bird's head into the insect cage, which was then closed. The cages with engorged flies were transported to the laboratory and held at 15 – 18° C, 70 ± 5 % RH and L/D photoperiod of 17:7 hr. Bowls with water were placed near each cage to maintain necessary relative humidity. The midges were supplied with 5–10 % saccharose solution; pads of cotton wool moistened in this solution were placed on the top of each insect cage daily.

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

The main methods were the same as described in the paragraph 2.2.7. Before dissection, biting midges were identified according to Gutsevich (1973). Engorged females were dissected daily in a drop of 0.85 % saline. The number of experimentally infected and dissected biting midges is given in (Table 2). Seventeen females were fed on uninfected common crossbill; they served as controls and were kept, dissected, and examined for ookinetes, oocysts, and sporozoites, as described above. Additionally, unfed females of *C. impunctatus* were collected and tested by PCR amplification for detection of natural infection prevalence with haemoproteids, as described below.

2.4.5. Microscopic examinations of preparations parasite morphology and statistical analysis

Main methods of the microscopic examinations of preparations and the parasite morphology investigation have been described in paragraph 2.2.8. The statistical analyses were carried out using the "Statistica 7" package (see paragraph 2.2.8), 95 % confidence limits of percentages are given in Table 2. Voucher specimens of ookinetes, oocysts and sporozoites of *H. minutus* (accession numbers 48810 – 48812 NS) and *H. belopolskyi* (48813 – 48815 NS) were deposited at the Nature Research Centre, Vilnius, Lithuania.

2.4.6. PCR - based examination

Total DNA was extracted from all samples using ammonium acetate extraction method. Other methods were as described in the paragraph 2.2.3. One negative control (nuclease - free water) and one positive control (one *H. minutus* microscopy positive blood sample, in the case of blood testing, and thoraxes of two flies experimentally infected with *H. minutus,* in the case of biting midge testing) were used per every 14 samples to control for false amplifications. No cases of false positive samples were found.

Fragments of DNA from the positive samples were sequenced from the 5' end with the primer HAEMF. The "Basic Local Alignment Search Tool" (National Centre for Biotechnology Information website: http://www.ncbi.nlm.nih.gov/BLAST) was used to determine lineages of detected DNA sequences, which were deposited in GenBank (accessions KJ627800 – KJ627802).

DNA extracted from individual flies was used to confirm the identification of *C. impunctatus* used in our experiments. For this purpose, the insect specific primers LCO149 and HCO2198 were applied to ampliphy a fragment of cytochrome oxydase subunit I of mitochondrial DNA (Folmer et al., 1994). All obtained sequences corresponded to the *C. impunctatus* DNA sequences, which are available in GenBank.

Because we used wild - caught *C. impunctatus* in experiments, it was essential to determine prevalence of natural *Haemoproteus* infection in the biting midges. Biting midges were collected at the same study site where we exposed donor birds to bites of *C. impunctatus* (see above). In all, 108 wild - caught females were tested by PCR amplification. DNA was extracted from 27 pools of biting midges, each containing 4 flies. Additionally, remains of all flies were collected after dissection; they were fixed in 96 % ethanol and tested by PCR amplification in order to confirm the identity of parasite lineages in experimentally infected flies.

2.5. Experiment 4: Determination peculiarities of the development of *Haemoproteus* parasites in blood - sucking mosquitoes (Culicidae)

2.5.1. Study site and collection of bird blood samples

This study was carried out at the Biological Station of Rybachy between 26 May and 4 July in 2012. Birds were caught and whole blood was taken as described in the paragraph 2.4.1.

2.5.2.Experimental design

Four siskins and four thrush nightingales with natural single infections of *H. tartakovskyi* and *H. balmorali*, respectively, were caught and kept in a bird housing facility. These birds were used as donors of *Haemoproteus* spp. gametocytes to infect mosquitoes. Four uninfected siskins were used as a source of parasite - free blood meal to feed control mosquitoes. All birds were taken from the wild and kept indoors as described in the paragraph 2.4.1. The blood for microscopic examination and PCR - based studies was taken from all birds as described above once each 2 or 3 days for approximately 1 month. During experimental infection of mosquitoes, the intensity of parasitemia was close to 1 % in all siskins and thrush nightingales, as determined by microscopic examination of blood films. Before mosquitoes were allowed to feed on infected birds, exflagellation was confirmed under a microscope as described by Valkiūnas et al. (2013c). The mosquito *O. cantans* is abundant at the study site and willingly takes blood meals on birds (Bernotienė, 2012).

To collect engorged mosquitoes, birds were held by hand and exposed to mosquito bites in the morning each day. This was carried out at a site located in bushes close to the Curonian Lagoon, approximately 30 m from the main building of the Biological Station. When two to three females began taking a blood meal on a bird's head, the head with feeding insects was carefully placed into an unzipped insect cage (approximately 12 × 12 × 12 cm) made of fine-mesh bolting silk. The engorged mosquitoes flew off after the blood meal. The cage with engorged mosquitoes was then closed using a zipper. Up to 10 mosquitoes were allowed to take blood meals on each bird daily. Cages with engorged females were transported to the laboratory and maintained in standard conditions (20 ± 1° C, 80 ± 2 % RH and natural photoperiod). Several pads of cotton - wool moistened with 10 % saccharose solution were placed in each cage to feed the mosquitoes. To control for presence of parasites, experimental and control insects were dissected daily between 1 and 17 dpi. Samples of infected and control insects were also fixed in 5 – 10 % neutral formalin on all dissection days to be used for histological examination. In all, we collected 304 mosquitoes fed on infected blood meals (146 individuals were infected with *H. tartakovskyi* and 158 with *H. balmorali*) and 90 mosquitoes fed on uninfected blood meal (controls). Of them, 91 infected mosquitoes were tested by PCR as detailed in Table 4; 109 infected and 40 control mosquitoes were dissected for microscopic examination

for ookinetes, oocysts and sporozoites to compare with the PCR - based results; 87 infected and 43 control mosquitoes were fixed for histology; 17 infected and 7 control insects survived to the end of the experiment and were not used in this study. Additionally, 160 mosquitoes were haphazardly collected unfed to determine the prevalence of natural infection with *Haemoproteus* parasites; they were tested by PCR - based methods, as described below.

Mosquitoes were processed individually for microscopic and PCR - based detection of parasites. Each mosquito body was carefully separated in three segments, the head, thorax and abdomen, under a microscope. To eliminate contamination of samples, we used new dissecting needle for each dissected mosquito.

2.5.3. PCR - based examination and sequencing

Two methods of total DNA extraction were applied. First, the standard phenol - chloroform method was used to extract genomic DNA from birds' whole blood. Second, we used innuPREP blood DNA Mini kit for extraction of DNA from the head, thorax and abdomen of mosquitoes (see paragraph 2.2.3).

We tested experimental mosquitoes for possible presence of bird DNA using PCR. The vertebrate - universal primers L14841 and H15149 (Kocher et al., 1989) were used to amplify a segment of 305 nucleotides of host cyt *b* gene. The Platinum Taq DNA polymerase (Life Technologies, NY) was used in the PCR reactions. One negative control (nuclease - free water) and one positive control (*H. tartakovskyi* microscopy - positive blood sample, in the case of blood testing, and thoraxes of two mosquitoes experimentally infected with *H. tartakovskyi*, in the case of mosquito testing) were used per every 12 samples to control for false amplifications. No case of false amplification was found.

All positive samples were sequenced in order to determine cyt *b* lineages of the detected parasite. Fragments were sequenced as described in paragraph 2.2.3. Detected sequences were deposited in GenBank (accessions KC559435 – KC559439).

Visualisation of "double bases" in electropherograms of cyt *b* sequences was used to estimate the presence of possible coinfections. The sequence divergence between different lineages was calculated using a Jukes - Cantor model of substitution (see paragraph 2.2.4). To determine the prevalence of natural *Haemoproteus* infection in *O. cantans* caught at the study site, DNA was extracted from

40 pools, each containing 4 mosquitoes; other protocols were the same as for individual mosquitoes.

2.5.4. Dissection of mosquitoes and making preparations of ookinetes, oocysts and sporozoites

Main methods were the same as described in the paragraph 2.2.7. Formalin - fixed heads, thoraxes and abdomens of 23 infected and 11 control mosquitoes on 1–6, 8, 10, 11, 12, 15 and 16 dpi were embedded in paraffin. Over 3000 histological sections of 4 μm were obtained, stained with hematoxylin - eosin and examined under a light microscop. Infected and control mosquitoes were processed and examined using the same methods.

2.5.5. Examination of preparations, voucher material and statistical analysis

The methods of microscopic examinations of preparations and investigation of parasites' morphology have been described in the paragraph 2.2.8. *Haemoproteu*s parasites were identified according to Valkiūnas (2005). All vector preparations were first examined at low magnification (× 100, × 200 and × 600) and then at high magnification (× 1000). The analyses were carried out using "Statistica 7" package (see paragraph 2.2.8). Percentages of infected insects were compared by Yates corrected $\chi 2$ test. Representative preparations of ookinetes (48702NS – 48706NS and 48712NS – 48717NS) and oocysts (48707NS – 48711NS and 48718NS – 48721NS) were deposited at the Nature Research Centre.

2.6. Experiment 5: Determination of virulence of *Haemoproteus* parasites for bird - biting mosquitoes and patterns of their development in these insects

2.6.1. Collection of blood samples

Material was collected, and experiments were carried out at the Biological Station in village Rybachy between 25 May and 1 July in 2013. Birds were caught and whole blood was taken as described in the paragraph 2.4.1.

2.6.2. Experimental design

In all, eight passeriform birds were caught, kept in a bird housing facility and used as sources of blood meals for mosquitoes. The birds were sorted out in three groups. First, one pied flycatcher (*Ficedula hypoleuca*), two siskins (*Carduelis*

spinus) and two red - backed shrikes (*Lanius collurio*) with high parasitaemia of different lineages of *H. balmorali* (lineage hCOLL3, parasitaemia 6.3 %), *H. tartakovskyi* (lineage hSISKIN1, 3.0 %) and *H. lanii* (lineages hRB1, 4.4 % and hRBS2, 9.6 %), respectively, were used as donors of *Haemoproteus* spp. gametocytes to infect mosquitoes. Second, one siskin infected with *H. tartakovskyi* (lineage hSISKIN1, 0.01 %) was used as a source of blood meal with light parasitaemia to feed mosquitoes. Third, two uninfected siskins were used as sources of parasite - free blood meals to feed control mosquitoes.

All birds were kept indoors as described in the paragraph 2.2.4. To control the level of parasitaemia, the blood for microscopic examination and PCR - based studies was taken from all birds before their exposure to mosquito bites. Before mosquitoes were allowed to feed on infected birds, exflagellation was confirmed under a microscope as described by Palinauskas et al. (2013b).

To collect engorged mosquitoes, we used methods described by Valkiūnas (2005). Briefly, birds were held by hands covered with ruby gloves and exposed to mosquito bites in the morning each day. This was done at the same site located in bushes close to the Curonian Lagoon, approximately 30 m from the main building of the biological station. When several females began taking a blood meal on a bird's head, the head with feeding insects was placed into an unzipped insect cage (approximately 45 × 45 × 45 cm). The engorged insects flew off after the blood meal. Cages with engorged females were transported to the laboratory and maintained as same as described in the paragraph 2.5.2. The mosquitoes were not allowed to re - feed on blood meal.

Fifty mosquitoes were exposed in each experiment. Only fully engorged females were used in this study. Dead and moribund mosquitoes were collected from cages daily, starting 12 hr post infection; they were identified according to Becker et al. (2003) and then counted and preserved for parasitological examination and PCR - based testing (see description below). The number of surviving mosquitoes was recorded daily. Length of all experiments was 20 dpi.

To detect parasites, we used the following protocol daily: (1) several dead insects from the control and all infected groups were dissected for observation of ookinetes, and their remnants were fixed individually in 96 % ethanol for PCR - based examination, and (2) entire bodies of remaining dead insects were fixed in 10 % neutral formalin and 96 % ethanol to be used for histological and PCR - based examinations, respectively. In the end of experiments, all survived mosquitoes were counted and fixed in 96 % ethanol. In all, we exposed, collected

and followed fates of 300 mosquitoes. Overall, 98 mosquitoes were examined for the presence of parasites in midgut preparations and histological section; presence of corresponding parasite lineages in infected mosquitoes was supported by PCR - based detection, as described below.

All insects were processed individually for microscopic and PCR - based examination. To eliminate contamination of samples, we used new dissecting needles for each dissected mosquito. All control mosquitoes were tested by PCR for the possible presence of natural infection in the end of this study. Additionally, 132 mosquitoes were haphazardly collected unfed on the study site, to determine the prevalence of natural infection with *Haemoproteus* parasites; they were tested byPCR - based methods, as described below.

2.6.3. PCR - based examination and sequencing

PCR - based examination is described in the paragraph 2.2.3 and in the paragraph 2.3.6. One negative control (nuclease free water) and one positive control (*H. tartakovskyi* microscopy - positive blood sample, in the case of blood testing, and thoraxes of two mosquitoes experimentally infected with *H. tartakovskyi*, in the case of mosquito testing) were used per every 10 samples to control for false amplifications. Visualization of "double bases" in electropherograms of cyt *b* sequences was used to estimate the presence of possible co-infections. The sequence divergence between different lineages was calculated using a Jukes - Cantor model of substitution see paragraph 2.5.3. Detected sequences were deposited in GenBank (accessions KF754352 – KF754355). To determine the prevalence of natural *Haemoproteus* infection in *O. cantans* caught at the study site, DNA was extracted from 66 pools, each containing two mosquitoes; other protocols were the same as for individual mosquitoes.

2.6.4. Dissection of mosquitoes and making preparations of parasites

The main methods were described in the paragraph 2.2.7. Dead and moribund mosquitoes were dissected for the presence of ookinetes daily between 0.5 and 4 dpi, as described by Valkiūnas (2005). Formalin - fixed heads, thoraxes and abdomens of mosquitoes were embedded in paraffin. In all, 380 histological sections of 4 μm were obtained, stained with hematoxylin - eosin and examined under a light microscope.

2.6.5. Examination of preparations, voucher material and statistical analysis

Examination of preparations was done as described in the paragraph 2.2.8. *Haemoproteus* parasites were identified according to Valkiūnas (2005). Intensity of parasitaemia was estimated before mosquitoes' exposure as a percentage (see paragraph 2.2.3). Because immature micro - and macrogametocytes (size of erythrocyte nucleus and less) do not exflagellate or produce macrogametes, respectively (Valkiūnas, 2005), such forms were not considered in the intensity estimates. All vector preparations were first examined at low magnification (× 100, × 600) and then at high magnification (× 1000).

The analyses were carried out using "Statistica 7" package. The Cox - Mantel's test was applied to estimate differences among cumulative numbers of survived mosquitoes in different groups. Percentages of survived insects on certain days were compared by Fisher's exact test. Representative preparations of ookinetes in midgut preparations (48758NS – 48760NS) and histological sections (48761NS – 48763NS) were deposited at the Nature Research Centre, Vilnius, Lithuania.

2.7. Experiment 6: Determination of development of *Plasmodium homocircumflexum* (lineage COLL4) in mosquitoes *Culex pipiens pipiens, Culex pipiens* form *molestus* and *Aedes vexans.*

2.7.1. Plasmodium homocircumflexum lineage and experimental infections of birds

We used *Plasmodium homocircumflexum* (mitochondrial cytochrome *b* lineage pCOLL4, GenBank accession no. KC884250), which was isolated from naturally infected red - backed shrike *Lanius collurio*. The birds were caught at the Biological Station in May 2010 and 2011, respectively. This parasite was multiplied in siskins and crossbills, and then cryopreserved in liquid nitrogen as described by Palinauskas et al. (2008). The experimental work was carried out at the Nature Research Centre between March 2012 and April 2013. Experimental birds (domestic canary) were commercially purchased and maintained in a vector - free room. Before experiments, the birds were kept in quarantine. All birds were tested for haemosporidian parasites both by microscopic and PCR - based examinations as described below. The cryopreserved strain of pCOLL4 were thawed and used to infect canaries as described by Palinauskas et al. (2008) with slight modifications.

For experimental investigation in total, 11 canaries were exposed to pCOLL4. We used pCOLL4 parasite lineage, which undergone 3 and 4 passages in wild birds after their original isolation, respectively.

2.7.2. Collection of blood from exposed canaries

To follow the development of parasitemia, the blood for microscopic and molecular examination was taken from all recipient canaries every three dpi for approximately one month (see paragraph 2.2.3).

2.7.3. Collection, maintenance and experimental infection of mosquitoes

We used *Culex pipiens pipiens* mosquitoes, which were collected hibernating from basements in Vilnius, Lithuania in January 2013. They were maintained in laboratory. After two weeks of laboratory adaptation, the wild - caught mosquitoes were used for experimental infection with malaria parasites. Part of these mosquitoes (104 mosquito females) were haphazardly chosen to determine the prevalence of natural malarial infection. They were tested using PCR - based methods as described by Kazlauskienė et al. (2013). *C. p. pipiens* f. *molestus* and *A. vexans* mosquitoes used in experiments were taken from mosquito colonies maintained in the laboratory as described in the paragraph 2.3.4.

Canaries with pCOLL4 gametocytaemia of approximately 0.1 – 2.5 % were placed in a mosquito cages (as described in the paragraph 2.2.6) and kept for approximately 1 hr once per 3 ‑ 4 days. Experiments with *C. p. pipiens*, *C. p. pipiens* f. *molestus* and *A. vexans* were carried out in parallel. Engorged females were taken from the experimental cages using an aspirator, placed in separate small insect cages (see paragraph 2.2.6) and dissected in intervals (see description below). Infected *C. p. pipiens* mosquitoes were kept until 25 days post infection (dpi) and dissected in intervals. Ookinetes preparations were made 1 – 4 dpi, oocysts preparations 6 – 25 dpi and sporozoites preparations 8 – 25 dpi. *C. p. pipiens* f. *molestus* mosquitoes were kept and dissected until 24 dpi with ookinetes, oocysts and sporozoites preparations at 1 – 3, 6 – 24 and 6 – 24 dpi, respectively. *A. vexans* mosquitoes were kept until 26dpi and then dissected for identification of sporogonic stages between 1 – 3, 6 – 26 and 9 – 26dpi, respectively. In total, we infected and dissected 86 mosquitoes (42 *C. p. pipiens*, 28 *C. p. pipiens* f. *molestus* and 16 *A. vexans*).

2.7.4. PCR - based examination

The total DNA was extracted from blood using the standard ammonium - acetate protocol see the paragraph 2.2.3. In second protocol we used outer primers HAEMF and HAEMR2 (Bensch et al., 2000), and the inner primers F144 and

R368 (Palinauskas et al., 2010) to amplify 224 bp fragment of the same cytochrome *b* gene. The thermal conditions for the second protocol were those described by Palinauskas et al. (2010). In both protocols for the first PCR we used 12.5 µl of Dreamtaq Master Mix (Fermentas, Lithuania), 8.5 µl of nuclease free water, 1 µl of each primer and 2 µl of template DNA. For the second PCR – 2 µl of the first PCR products were used as a template. The conditions for the second PCR were the same as the first, except 35 cycles instead of 20 were run. The amplification success was evaluated by using MultiNa electrophoresis system (Shimadzu, Japan). One negative control (nuclease - free water) and 1 positive control (microscopically proved positive sample with *Plasmodium* sp. infection) were used once every 7 samples to control for false amplifications, as described in the paragraph 2.2.3.

2.7.5. Microscopy, morphological analysis of parasites and statistical analysis

Examination of preparations was done as described in paragraph 2.1.8. Parasites were identified according to Valkiūnas (2005). Student's t - test for independent samples was used to determine statistical significance between mean linear parameters. Representative preparations of ookinetes (48778 – 48780 NS) and oocysts (48781 – 48783 NS) were deposited at the Nature Research Centre, Vilnius, Lithuania.

2.7.6. Dissection of mosquitoes and preparations of ookinetes, oocysts and sporozoites specimens

We used the same methods as described in the paragraph 2.1.7.

3. RESULTS AND DISCUSSION

3.1. Experiement 1. Patterns of development of the closely related lineages pSGS1 and pGRW11 of *Plasmodium relictum* and different isolate of the lineage pSGS1 in the blood sucking mosquitoes *Culex pipiens pipiens* (Culicidae)

3.1.1. Results

According both to PCR - based analysis and microscopic examination of blood films, malarial parasites were absent from all canaries prior to infection, and all negative controls remained uninfected throughout the course of the experiment.

Parasitemia developed in all experimentally infected canaries, with mean prepatent period of approximately 5 dpi for all strains. Primary parasitemia was light at acute stage (< 3 %) in all canaries; it decreased rapidly and maintained at low level (<1 %) throughout the course of the experiment. Mosquito feeding was carried out 20 days after the onset of the infection, to coincide with the chronic parasitemia. Canaries with gametocyte parasitemia between 0.01 and 0.1 % were used as donors to infect mosquitoes.

The restriction enzyme assay revealed that all tested mosquitoes belong to *C. pipiens*, as described by Hesson et al. (2010), Kazlauskienė et al. (2013). Examination of genitalia morphology of laboratory reared mosquitoes showed the same result. PCR - based analysis identified *C. p. pipiens* because the positive amplification was obtained only with primers ACEpip and ACEpip2, which are specific for this subspecies. The siphonal index of hatched larvae was 3.8 (Vinogradova, 2000), which is typical of *C. p. pipiens*. Additionally, we observed strict eurygamy (an inability of mosquitoes to mate in a small cages) of the captivity - reared imago. These observations reject an opportunity of presence of *C. pipiens* form *molestus* in our experiments. We thus conclude that all experimental mosquitoes were *C. p. pipiens.*

PCR - based detection did not reveal natural malarial infections in 114 wild - caught hibernating mosquitoes, indicating that mosquitoes collected in basements and used in our experiments were malaria - free. All experimental mosquitoes were susceptible to infection with 3 strains of *P. relictum*; sporogonic stages were observed in 100 % of experimentally infected and dissected females. Ookinetes, oocysts and sporozoites of all strains (Fig. 4 a – i) were reported in mosquitoes at

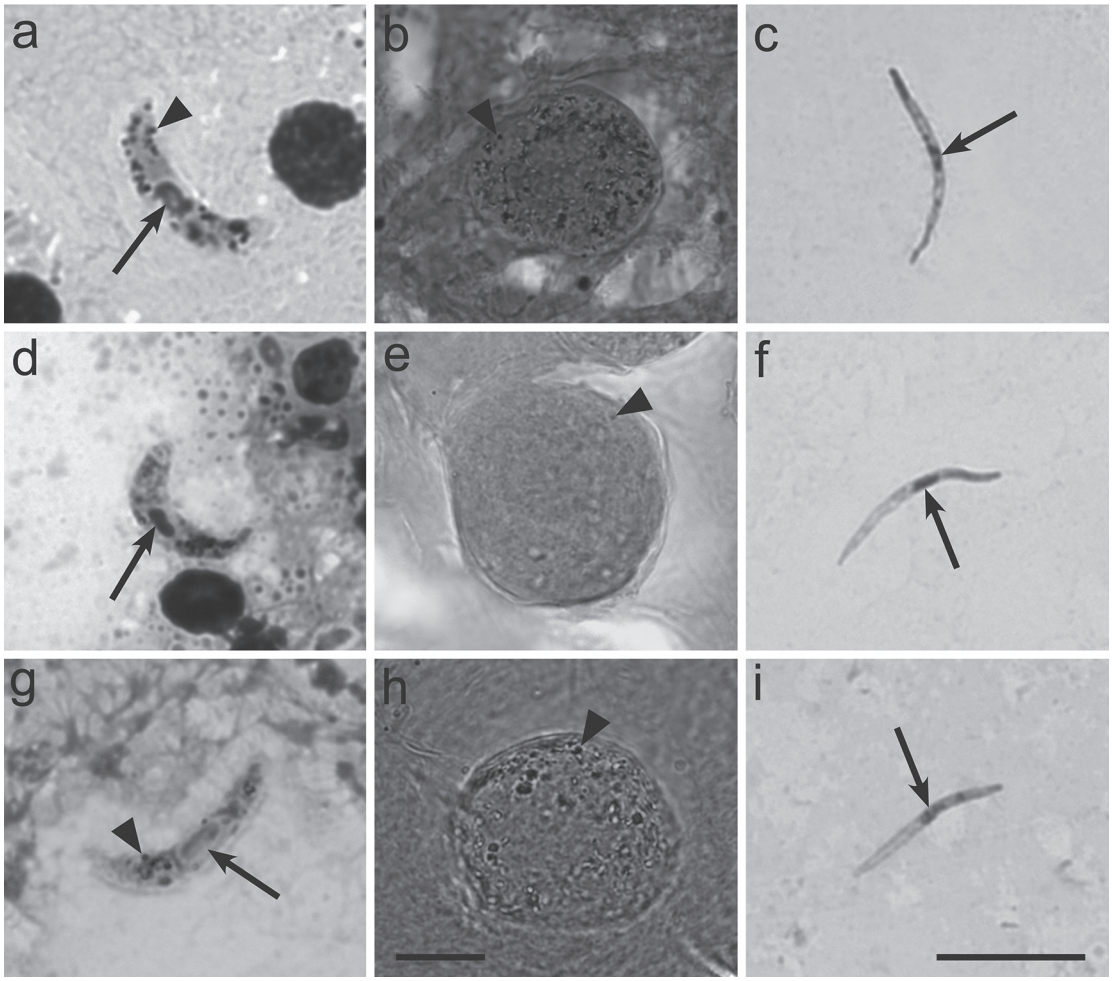

Fig 4. Ookinetes (a, d, g), oocysts (b, e, h) and sporozoites (c, f, i) of *Plasmodium relictum* in mosquitoes *Culex pipiens pipiens* experimentally infected with the parasite lineage pSGS1 from *Loxia curvirostra* (a – c), lineage pSGS1 from *Serinus canaria domestica* (d – f) and lineage pGRW11 from *Passer domesticus* (g – i). Methanol - fixed and Giemsa - stained thin films (a, c, d, f, g, i). Formalin - fixed whole mounts stained with Erlich's hematoxylin (b, e, h). Long simple arrows – nuclei of parasites; triangle arrowheads – pigment granules. Scale bars = 10 μm; long bar is for ookinete and sporozoite images, and short bar is for oocyst images.

the same dpi. In other words, the pattern of sporogonic development of these 3 lineages was the same in *C. p. pipiens*. For all parasite strains, 1) ookinetes were seen on 1 and 2 dpi, their number markedly decreases on 3 dpi, and the parasites are absent from midgut on 6 dpi; 2) growing oocysts are numerous in the midgut on 6 dpi, they develop markedly asynchronously; 3) mature oocysts were seen until 24 dpi, and they degraded between 24 and 26 dpi, and not seen on 28 and 32 dpi; 4) sporozoites were first reported in the salivary glands on 14 dpi and were seen until the end of the experiment (32 dpi). There were no morphological or morphometric differences discernable among mature ookinetes (Fig. 4 a, d, g), mature oocysts (Fig. 4 b, e, h) or sporozoites (Fig. 4 c, f, i) among 3 tested parasite strains (Table 1, $P < 0.05$ for all corresponding data).

Table 1.
Morphometry of sporogonic stages of *Plasmodium relictum* (lineages pSGS1 and pGRW11) in *Culex pipien pipiens*.

Feature	Measurements [a]		
	Lineage pSGS1		Lineage pGRW11
	Strain from *Loxia curvirostra*	Strain from *Serinus canaria domestica*	Strain from *Passer domesticus*
Mature ookinete			
Length	12.4-20.3 (15.9±2.2)	14.9-19.4 (16.8±1.5)	9.8-19.1 (15.9±2.6)
Width	1.5-2.7 (2.1±0.3)	1.5-2.5 (2.2±0.3)	1.4-2.8 (1.9±0.5)
Area	27.5-45.9 (34.5±4.6)	33.9-44.8 (37.4±3.7)	20.6-39.5 (31.7±5.9)
Mature oocyst [b]			
Minimum diameter	32.8-49.3 (41.9±3.3)	24.0-51.6 (35.7±8.3)	21.7-61.4 (35.5±9.2)
Maximum diameter	40.5-57.1 (45.7±3.6)	31.2-52.5 (38.4±7.4)	23.7-64.3 (40.0±11.8)
Area	152.7-187.7(167.1±8.4)	107.6-196.4 (141.7±26.7)	99.8-243.4 (141.4±36.1)
Sporozoite			
Length	13.8-17.8 (14.2±1.5)	11.1-16.1 (13.9±1.4)	11.9-16.8 (13.9±1.7)
Width	0.7-1.1 (0.9±0.1)	0.7-1.1 (0.9±0.1)	0.8-1.3 (1.0±0.1)
Area	27.9-38.8 (32.8±2.9)	24.2-34.3 (30.2±2.7)	23.2-34.9 (29.7±3.4)

[a]Measurements of oocysts (n = 21, formalin - fixed preparations), sporozoites (n = 21, methanol - fixed preparations), and ookinetes (n = 10, methanol - fixed preparations) are given in micrometers. Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation.

3.1.2. Discussion

Recent molecular studies have revealed an astonishing genetic diversity of avian malaria parasites and related haemosporidians (Haemosporida), indicating that the number of their genetic lineages, which can be considered as independent evolutionary entities might be higher than the number of vertebrate host species (Bensch et al., 2004; Ricklefs et al., 2004). Surprisingly, nearly each bird species is infected with different haemosporidian parasite lineage and some closely related lineages can co - exist in the same individual host without evidence of recombination (Perez-Tris and Bensch, 2005; Jarvi et al., 2008). It is unclear how many of such lineages are biological species and how many of them are adaptive clones or isolates of the same parasite species, thereby representing different levels of intraspecific variation. It is also unclear how such lineages might evolve, interact and maintain in the same ecosystems. Additional information is needed to answer these questions.

Due to application of PCR - based methods, the field of molecular biology of avian haemosporidians has been rapidly developing (Martinsen et al., 2008; Bensch et al., 2009; Ricklefs and Outlaw, 2010; Marzal et al., 2011). However, a few studies address patterns of development of wildlife haemosporidian parasites in avian hosts and vectors (Palinauskas et al., 2008; Zehtindjiev et al., 2008; Vezilier

et al., 2010). Differences among numerous genetic lineages of haemosporidians in terms of their life history strategies remain unclear. That leads to the markedly asymmetrical knowledge between the rapidly accumulating data about parasites' DNA sequences, on the one hand and the basic biology of haemosporidians, on the other hand. This is unfortunate because linkage between the DNA sequence data and the basic biology information can provide important knowledge for epidemiology and would be helpful for better understanding phylogenetic relationships of these organisms.

Several studies addressed *P. relictum* cyt *b* lineages in terms of their distribution by hosts and geographical regions (Beadell et al., 2006; Beadell et al., 2009; Dimitrov et al., 2010; Marzal et al., 2011), virulence for birds (Palinauskas et al., 2008; Cellier-Holzem et al., 2010), sporogony (Vezilier et al., 2010), but comparative studies about sporogonic development of different lineages of this parasite (and other avian malaria parasites too) in the same mosquito species are lacking. It remains unclear if there are differences in sporogony patterns of different lineages and isolates of the same lineage in the same mosquito species.

Because lineages pSGS1 and pGRW11 are widespread in the Old World, often transmitted in sympatry and also are indistinguishable based on morphology of their blood stages (Palinauskas et al., 2007; Bensch et al., 2009; Marzal et al., 2011), we carried out this study for better understanding biology of these parasite lineages. This study shows that *C. p. pipiens* is susceptible to 3 isolates of 2 lineages of *P. relictum*. Additionally, *C. p. pipiens* transfer viable sporozoites of the lineage pSGS1 to canaries trough biting. The latter observation is important because presence of *Plasmodium* spp. sporozoites in salivary glands does not necessarily means that salivary - gland sporozoites are infective for avian hosts or mosquitoes can transfer them through biting (Nayar et al., 1980; Valkiūnas, 2005). Investigation of sporogony of wildlife haemosporidians is time and funding consuming, thus is hardly possible to carry out with many parasite lineages. It would be helpful to use the phylogenetic data (Fig. 5) for predicting sporogonic development of different lineages.

The key results of this study are that (1) the closely related lineages of *P. relictum* and (2) the different isolate of same lineage develop synchronously and produce morphologically indistinguishable sporogonic stages and infective sporozoites in *C. p. pipiens*. We predict that this might be true for closely related lineages of other malaria parasite morphospecies, for instance the genetically similar lineages of *P. relictum, P. circumflexum, P. elongatum* and some other

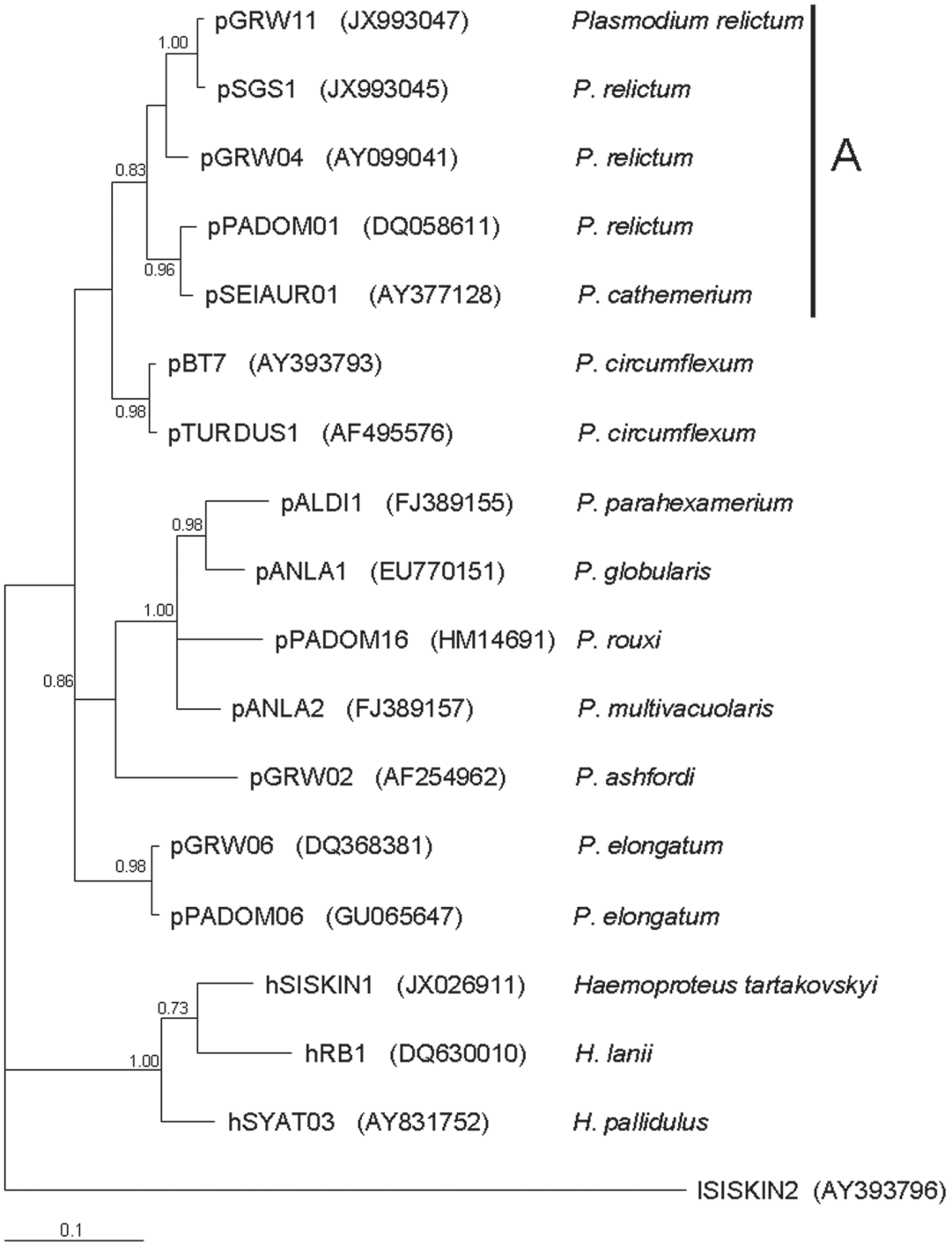

Fig 5. Bayesian phylogeny of mitochondrial cytochrome *b* lineages (478 bp) of avian *Plasmodium* spp. (14 sequences) and *Haemoproteus* spp. (3 sequences). One lineage of *Leucocytozoon* sp. is used as outgroup. Posterior probabilities are indicated near the nodes. Codes of lineages and GenBank accessions (in parenthesis) are given before parasite species name. Vertical bar A indicates lineages pSGS1, pGRW11 and other closely related lineages of *Plasmodium relictum* and *P. cathemerium*.

haemosporidians (see Fig. 5). In othe words, this study indicated that phylogenetic trees based on cyt *b* gene sequences might be helpful for predicting sporogonic development of closely related lineages in vectors. This assumption is in parallel to the Martinsen et al.'s (2008) study suggesting that evolution of major

groups of haemosporidian parasites is closely associated with adaptation to certain groups of dipteran vectors. Data of the present study indicate that the same association also might be present in clades of genetically similar lineages on the morphospecies level of haemosporidians; that warrants further investigation. These data are helpful for better understanding biology and diversity of avian *Plasmodium* spp., indicating possible directions for predicting sporogony patterns of numerous genetically similar lineages of *Plasmodium* spp., which sporogonic development and vectors remain unknown. The important next step in these studies should be comparative investigation on sporogony of other closely related *P. relictum* lineages and *P. cathemerium* (Fig. 5, clade A) in *C. p. pipiens* at the same laboratory conditions.

3.2. Experiment 2. The developmend of *Plasmodium relictum* (pSGS1 and pGRW11) in mosquito *Culex pipiens pipiens* form *molestus*

3.2.1. Results

All control birds remained uninfected throughout the course of the experiment, as confirmed both by microscopic examination of blood films and PCR - based testing. Ookinetes of the lineages pSGS1 and pGRW11 of *P. relictum* were detected in the midgut preparations of all corresponding exposed mosquito groups 1 – 2 dpi (Fig. 6 a, d). Oocysts of both lineages were seen in the midgut

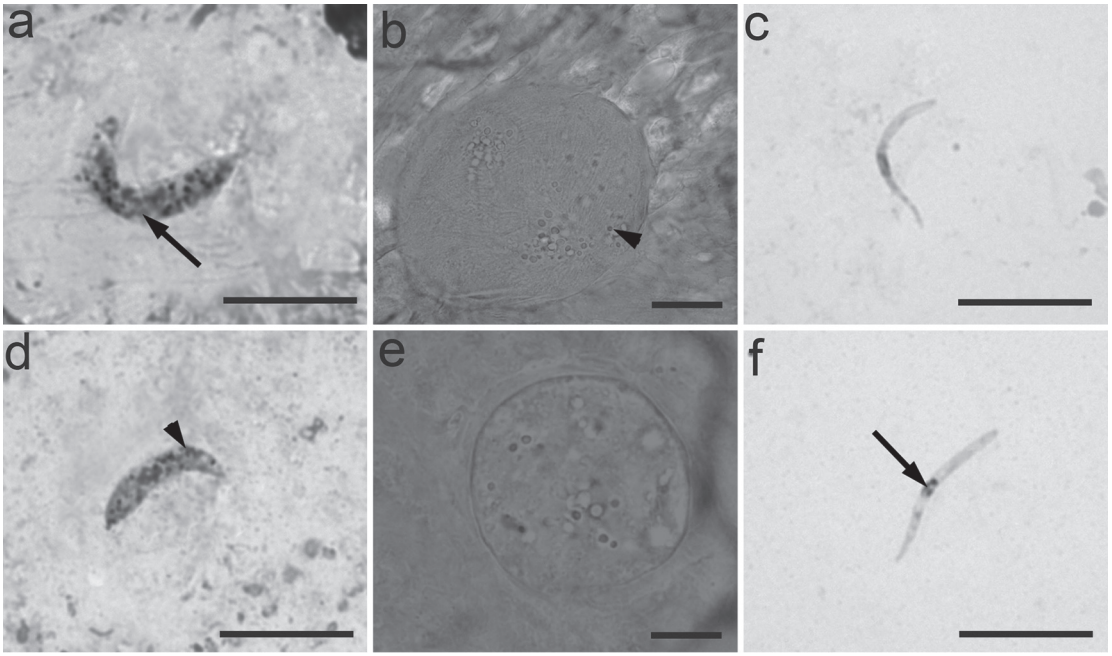

Fig 6. Ookinetes (a, d), oocysts (b, e) and sporozoites (c, f) the lineages pSGS1 (a – c) and pGRW11 (d – f) of *Plasmodium relictum* in experimentally infected mosquitoes *Culex pipiens pipiens* form *molestus*. Long simple arrows – nuclei of parasite; triangle arrowheads – pigment granules. Scale bars = 10μm.

of all infected individual mosquitoes 10 – 15 dpi (Fig. 6 b, e). In both parasite lineages, there was marked variation in oocyst numbers in different individual mosquitoes (Fig. 7). However, the lineage pGRW11 developed significantly greater numbers of oocysts than the lineage pSGS1 ($P = 0.004$). Sporogony of both lineages completed and sporozoites were observed in mosquito salivary glands 15 – 18 dpi; they were seen in all salivary gland preparations (Fig. 6 c, f), indicating synchronous sporogonic development of both lineages under the same conditions. Sequencing confirmed the presence of the lineages pSGS1 and pGRW11 of *P. relictum* in 2 different experimental mosquito groups, in which sporozoites were observed. Morphology of ookinetes, oocysts and sporozoites of both lineages was similar (Fig. 6); the sporogonic stages of the same morphology developed in *C. p. pipiens* (see section 3.1.1).

3.2.2. Discussion

There are 3 key results of this study. First, we show that *C. p. pipiens* f. *molestus* mosquitoes readily take blood meals on birds in spite of established opinion about high degree of anthropophily of this form (Becker et al., 2003). This mosquito should be involved in natural transmission of avian malaria, particularly in human settlements where this form is often abundant (Vinogradova et al., 1996) and transmission of avian malaria often occurs (Valkiūnas, 2005).

Second, *C. p. pipiens* f. *molestus* should be considered in epidemiology studies of avian malaria because the lineages pSGS1 and pGRW11 of *P. relictum* successfully complete sporogony in this fly.

This finding is in accordance with our former observation about successful synchronous sporogonic development of the same lineages in *C. p. pipiens* mosquitoes (Kazlauskienė et al., 2013, see chapter 3.1.1). Both mosquitoes can act as vectors of *P. relictum* (Valkiūnas, 2005; Vézilier et al., 2010; Santiago-Alarcon et al., 2012; this study), but epidemiological significance of *C. p. pipiens* f. *molestus* has been insufficiently addressed in avian malaria studies.

Third, the susceptibility of *C. p. pipiens* f. *molestus* to the closely related lineages pSGS1 and pGRW11 of *P. relictum* was different, with different oocyst burden in the exposed insects (Fig. 7). Gametocytemia of both lineages in donor canaries was similar in both experiments. It remains unclear why significantly greater number of oocysts develops during sporogony of the lineages pGRW11. This finding is interesting because genetic difference in cyt *b* gene sequences between these 2 parasite strains is negligible (1 base pair), indicating that spo-

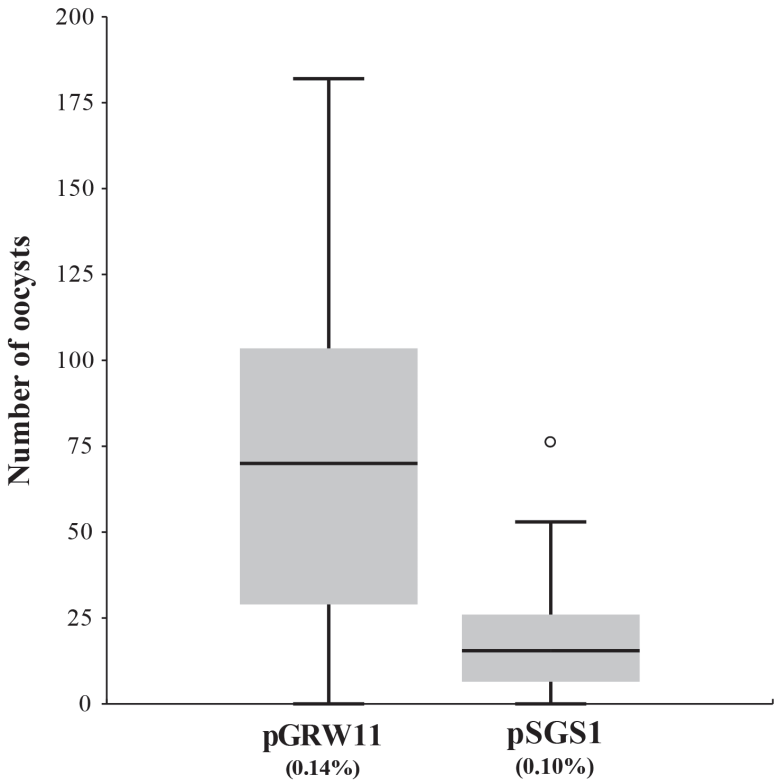

Fig 7. Oocyst burden of 2 lineages of *Plasmodium relictum* (pSGS1 and pGRW11) in experimentally infected mosquitoes *Culex pipiens pipiens* form *molestus*. Domestic canaries were used as donors of gametocytes (gametocytaemia at the day of the mosquito feeding is indicated in parentheses). Twenty flies were exposed to each parasite lineage. The median number of oocysts (horizontal black bars) is shown. Gray boxes below and above the median indicate the first and third quartiles, respectively. Vertical lines delimit non - outlier ranges on both sides of the boxes. One individual count (lineage pSGS1) is considered outlier and marked as a dot.

rogony success can be markedly different even in closely related lineages of the same malaria parasite in the same mosquito species. Oocyst number is directly related to the number of transmissible sporozoites that develop in the salivary glands, and also to the mosquito longevity (Sinden et al., 2007; Dawes et al., 2009). That should influence the mosquito vectorial capacity, but remains insufficiently investigated in avian malaria parasites, particularly on the level of different lineages of the same species. This study provides first information about patterns of sporogonic development of the lineages pSGS1 and pGRW11 in *C. p. pipiens* f. *molestus*.

It is important to note that Garnham (1966) reported complete sporogony of *P. garnhami* in *C. p. pipiens* f. *molestus*, but susceptibility of different colonies was markedly different: mosquitoes of one colony were entirely insusceptible to the parasite, but the other readily took the infection, with sporozoites present in salivary glands on the 13th dpi at 27° C. It is thus important to test and use different geographical isolates of this insect in avian malaria studies. *C. p. pipiens*

f. *molestus* is a convenient model for such research. Because *C. p. pipiens* f. *molestus* (1) readily takes blood meal on birds, (2) is susceptible to the widespread lineages of *P. relictum,* and (3) is easy to breed under laboratory conditions using wild-sampled eggs, larvae or imago (Vinogradova et al., 1996; Becker et al., 2003), we recommend this form for the experimental research with avian malaria parasites. *P. relictum* is the most common avian malaria parasite found in birds belonging to many orders, but is particularly prevalent in passerines (Bishop and Bennett, 1992; Valkiūnas, 2005).

Numerous mosquito species belonging to different genera transmit avian malaria. According to available experimental data, *P. relictum* completes sporogony in over 25 mosquito species belonging to *Aedes, Anopheles, Culex* and *Culiseta*, however *Culex* mosquitoes are the most effective vectors (Corradetti et al., 1970; Valkiūnas, 2005; LaPointe et al., 2010; Santiago-Alarcon et al., 2012; Cornet et al., 2013). It remains unclear if all described lineages of this parasite complete sporogony in mosquitoes belonging to different genera and what are imputes of these mosquito species in transmission of avian malaria in wildlife; additional studies are needed for better understanding this issue.

3.3. Experiment 3. Determination of the vectors of *Haemoproteus minutus* and *H. belopolskyi*

3.3.1. Results

Microscopic identification and PCR - based testing showed that all experimental flies belonged to *C. impunctatus.* According to PCR - based analysis, no natural infection was found in wild - caught biting midges. Parasites were also not detected in control flies (Table 2) both by PCR and microscopic methods. *H. minutus* and *H. belopolskyi* completed sporogony in *C. impunctatus*. In accordance to microscopic observation, the PCR and sequencing confirmed the presence of corresponding parasite lineages in experimentally infected biting midges (Table 2). Mature ookinetes of *H. minutus* were seen in the midgut contents of experimentally infected flies between 1 and 4 hr post infection (hr pi), and they were not seen 6 hr pi, indicating blisteringly rapid development and movement of the parasites in the midgut. Ookinetes of *H. belopolskyi* developed more slowly; they were seen 1 dpi and also reported 3 dpi. No difference was discernable in the percentage of midges with ookinetes between two parasite species ($P = 0.06$, Table 2). Ookinetes of both species were elongate wormlike

Table 2.
Percentages of experimentally infected *Culicoides impunctatus* with two species of *Haemoproteus.*

Sporogonic stage	Number of engorged flies / number of infected flies		
	Control	*H. minutus*	*H. belopolskyi*
Ookinete	5/0 (0, 0.0 – 18.7)[a]	26/21 (80.7, 64.5 – 97.0)	18/18 (100, 94.5 – 100)
Oocyst	6/0 (0, 0.0 – 15.8)	15/15 (100, 93.5 – 100)	11/11 (100, 80.1 – 100)
Sporozoite	6/0 (0, 0.0 – 15.8)	28/23 (82.1, 67.0 – 97.3)	12/11 (91.7, 73.3 – 100)
Total	17/0 (0, 0.0 – 5.8)	69/59 (85.5, 88.0 – 94.0)	41/40 (97.5, 92.6 – 100)

[a] Percentages are given in parentheses, followed by 95 % confidence limits of the percentage. All infections were determined by microscopic examination of preparations, except for control group, which was also tested by PCR - based methods.

Table 3.
Morphometry of ookinetes, oocysts and sporozoites of two species of *Haemoproteus* in *Culicoides impunctatus.*

Feature	Measurements[a]	
	H. minutus	*H. belopolskyi*
Ookinete		
Length	5.7-9.1 (7.6±0.8)	11.5-20.8 (16.5±2.5)
Width	1.2-2.9 (2.1±0.4)	1.6-2.5 (2.0±0.2)
Area	7.9-19.5 (12.7±3.3)	17.5-34.2 (26.6±4.5)
Area of nucleus	1.0-2.7 (1.7±0.5)	2.0-4.5 (3.3±0.6)
Oocyst		
Minimum diameter	3.7-6.8 (5.0±0.9)	3.9-6.9 (4.8±0.8)
Maximum diameter	4.1-7.2 (5.6±0.9)	4.4-7.6 (5.5±1.0)
Area	14.1-36.8 (23.8±7.8)	15.2-42.8 (23.0±9.6)
Sporozoite		
Length	10.9-14.5 (13.2±1.1)	7.6-10.6 (9.0±0.7)
Width	1.0-1.4 (1.1±0.1)	0.8-1.3 (1.0±0.1)
Area	9.3-15.7 (12.6±1.7)	5.6-10.4 (7.3±1.1)
Area of nucleus	0.7-1.2 (0.9±0.1)	0.7-1.5 (1.1±0.2)

[a] Measurements of ookinetes (n = 21, methanol - fixed preparations), oocysts (n = 12, formalin-fixed preparations) and sporozoites (n = 21, methanol - fixed preparations) are given in micrometers. Minimum and maximum values are provided, followed in parentheses by the arithmetic mean and standard deviation.

bodies with prominent, slightly of center located nuclei and visible vacuoles (Fig. 8 e, f). Occasionally, a few pigment granules were discernable in the cytoplasm of ookinetes of both parasite species (Fig. 8 e). There was a significant difference in the length and area of ookinetes of two parasites (Table 3). Ookinetes of *H. belopolskyi* were significantly longer and greater in area than those of *H. minutus* ($P < 0.001$, both for length and area). Oocysts both of *H. minutus* and *H. belopolskyi* were first seen in the midgut wall 3 dpi, and they were reported in the midgut preparations until 6 dpi. No difference was discernable in the percentage of midges with oocysts between two parasite species ($P = 1$, Table 2).

In formalin fixed preparations, oocysts appeared as small roundish bodies (Fig. 8 g, h, Table 3). Pigment was visible in some oocysts. There was no signifi-

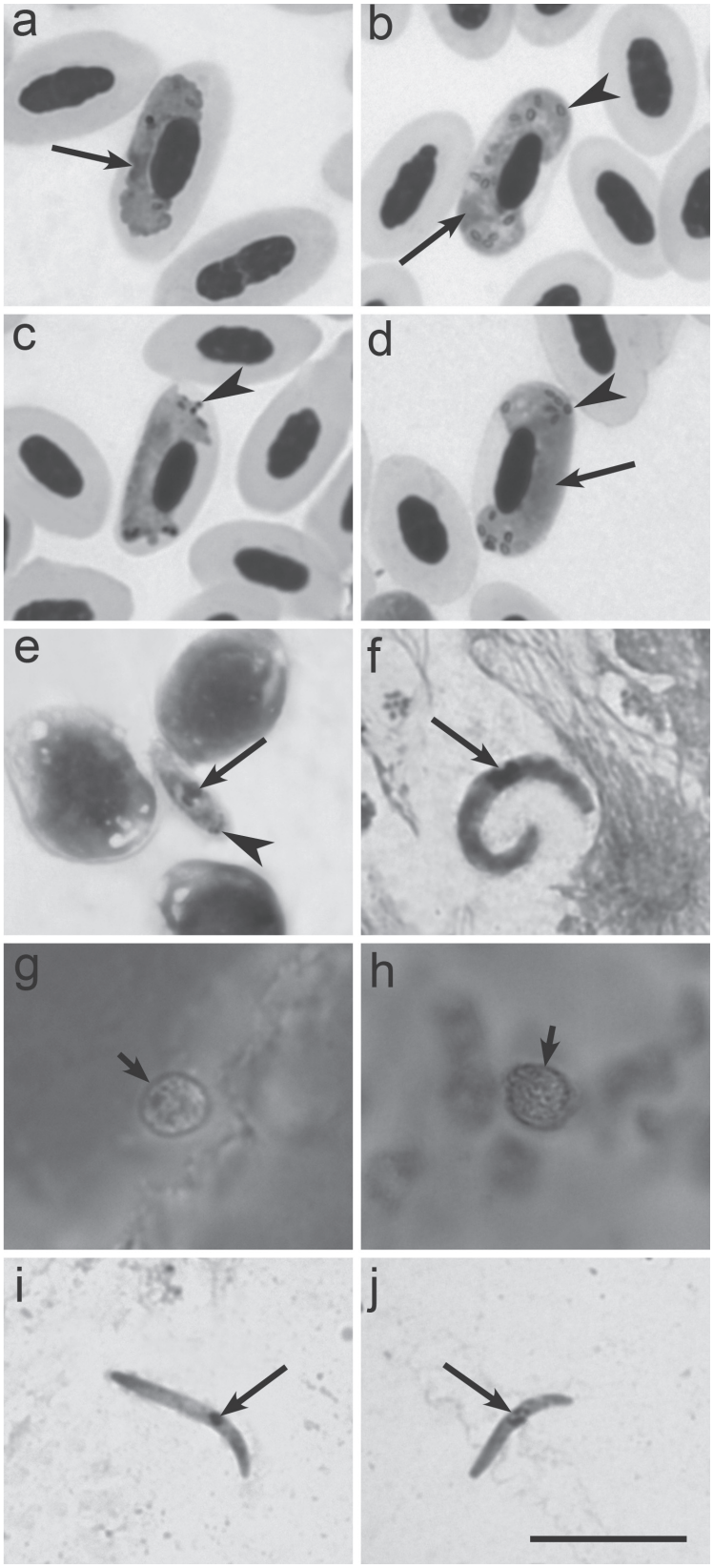

Fig 8. Gametocytes (a – d) and sporogonic stages (e – j) of *Haemoproteus minutus* (a, c, e, g, i) and *H. belopolskyi* (b, d, f, h, j): mature macrogametocytes (a, b) and microgametocytes (c, d) in the peripheral blood of donor birds before experimental infection of biting midges *Culicoides impunctatus* Ookinetes (e, f), oocysts (g, h), sporozoites (i, j). Methanol - fixed and Giemsa - stained thin films (a – d, e, f, i, j). Formalin - fixed whole mounts stained with Erlich's hematoxylin (g, h). Long simple arrows – nuclei of parasites, arrowheads – pigment granules, short simple arrows – oocysts. Scale bar = 10 μm.

cant difference in the diameter or area of oocysts between these parasite species 4 dpi ($P > 0.8$, both for diameter and area). Sporozoites of *H. minutus* and *H. belopolskyi* were seen in the salivary glands of the biting midges 7 dpi. They were reported in salivary gland preparations of the majority of dissected insects between 8 and 12 dpi (the period of observation). No difference was discernable in the percentage of midges with sporozoites between two parasite species ($P = 0.41$, Table 2). The parasites were fusiform bodies with slightly of center located nuclei and approximately equally pointed ends (Fig. 8 i, j). Sporozoites of *H. belopolskyi* were significantly shorter than those of *H. minutus* ($P < 0.001$, Table 3). It worth mentioning that sporozoites were significantly longer than ookinetes in *H. minutus* ($P < 0.001$, Table 3, Fig.8 e, i), but this was not a case in *H. belopolskyi*, in which sporozoites were significantly shorter than ookinetes ($P < 0.001$, Table 3, Fig. 8 f, j).

3.3.2. Discussion

The key result of this study is that two species of widespread *Haemoproteus* parasites, i. e. *H. minutus* (lineage hTURDUS2) and *H. belopolskyi* (hHIICT1) complete sporogony and produce sporozoites in *C. impunctatus*. This biting midge is common in Europe and is abundant at our study site (Glukhova and Valkiūnas, 1993; Liutkevičius, 2000; Valkiūnas et al., 2002). Sporogony of six species of *Haemoproteus* completes in *C. impunctatus*; these are *H. balmorali*, *H. dolniki*, *H. fringillae*, *H. lanii, H. parabelopolskyi* and *H. tartakovskyi* (Valkiūnas et al., 2002; Valkiūnas, 2005).

The present study adds two species to the group of avian haemoproteids, which produce sporozoites in *C. impunctatus.* Former experimental studies proved that infective sporozoites of *H. fringillae* and *H. parabelopolskyi* developed in *C. impunctatus* (Valkiūnas and Iezhova, 2004; Valkiūnas, 2005), indicating that this fly is an important vector of avian haemoproteids and worth more attention in epidemiological studies of avian haemoproteosis. The present study supports former conclusions about important role of biting midges of *Culicoides* in transmission of avian *Haemoproteus* spp. (Garnham, 1966; Atkinson, 2008). It worth mentioning that several molecular studies detected DNA of *Haemoproteus* spp. in mosquitoes and speculated about possible involvement of these insects in haemoproteid transmission (Ishtiaq et al., 2008; Kimura et al., 2010; Njabo et al., 2011; Kim and Tsuda, 2012; Ventim et al., 2012). Recent experimen-

tal research shows that this speculation likely is incorrect due to DNA amplification of *Haemoproteus* parasites, which abort development and persist in resistant insects, sometimes as long as two weeks (Valkiūnas et al., 2013c). Because haemosporidians have complicated life cycles and their abortive infections are common, demonstration of sporozoites in blood - sucking insects is essential for definitively demonstrating the insects are vectors.

The pattern of sporogony of *H. balmorali*, *H. belopolskyi, H. dolniki*, *H. fringillae*, *H. lanii, H. minutus, H. parabelopolskyi* and *H. tartakovskyi* in *C. impunctatus* is similar at same conditions (Valkiūnas, 2005; this study). Mainly, mature ookinetes first seen in midguts approximately 1 dpi and oocysts 3 dpi, and sporozoites reported in salivary glands 5 – 7 dpi at 15 – 18° C. Rapid sporogony has been also reported in *H. mansoni* (Fallis and Bennett, 1960; Atkinson, 1991a). An exception is *H. minutus*, which mature ookinetes develop within several hours after infection in biting midges.

Interestingly, ookinetes of this parasite develop more rapidly than any other species of haemosporidian parasites (Garnham, 1966; Sinden, 1998; Valkiūnas, 2005). Biological meaning of such rapid development of ookinetes is unclear. Tiny size probably contributes to the rapid ookinete development of this parasite (Table 3).

It worth mentioning that the rate of maturation and the morphology of *H. minutus* ookinetes were the same during development both in vivo and in vitro (Valkiūnas, 2005; this study). Tiny ookinetes, which are similar to *H. minutus,* have been described only in *Haemoproteus pallidus*, but vectors of the later parasite remain unknown. Ookinetes of other investigated *Haemoproteus* spp. are approximately 2 – fold longer than those of *H. minutus* and *H. pallidus* (Valkiūnas et al., 2002; Valkiūnas, 2005). Interestingly, in spite of marked size differences between ookinetes of *H. minutus* on the one hand, and ookinetes of *H. belopolskyi* and other investigated *Haemoproteus* spp. on the other hand, the size and the rate of maturation of oocysts of all investigated *Haemoproteus* species are similar in *C. impunctatus*. Further studies are needed for better understanding this phenomenon. It worth mentioning that both ends of sporozoites are approximately equally pointed in *H. minutus* and *H. belopolskyi* (Fig. 8, i, j); this is a diagnostic feature of haemoproteid species belonging to subgenus *Parahaemoproteus* (Fallis and Bennett, 1960; Garnham, 1966; Atkinson, 1991). This feature is readily distinguishable in salivary gland preparations in all investigated species of *Parahaemoproteus* (Valkiūnas, 2005) and can be used in determining subge-

neric identity of parasites based on morphology of sporozoites. Species of subgenus *Haemoproteus* are transmitted by louse flies; these parasites produce large (> 20 μm in diameter) oocysts, in which develop sporozoites with one end more pointed than the other end (Garnham, 1966; Baker, 1966; Atkinson, 2008).

H. minutus is widespread, prevalent and relatively benign in common blackbirds in Europe, but it kills several species of captive parrots on the stage of tissue megalomeronts, if these birds are exposed to the infection (Olias et al., 2011; Palinauskas et al., 2013a). This study shows that *C. impunctatus* transmits *H. minutus* and seven other haemoproteid species. This biting midge is important vector of avian *Haemoproteus* parasites and worth attention during development of prevention measures against avian haemoproteosis.

3.4. Experiment 4. Development of *Haemoproteus* parasites in blood - sucking mosquitoes (Culicidae)

3.4.1. Results

The mitochondrial cyt *b* lineage hSISKIN1 of *H.tartakovskyi* and the lineage hROBIN1 of *H. balmorali* were present in all donor siskins and thrush nightingales, respectively. The genetic distance in cyt *b* gene between these parasites is 4.3 %. Single infections were present in the donor birds, as confirmed both by microscopic examination and PCR - based detection. The same lineages were reported in experimentally infected mosquitoes. We assume all isolates of *H. tartakovskyi* and *H. balmorali* from different individual donor birds to be biologically identical because isolates (1) show morphologically indistinguishable blood stages (Fig. 9 a – d), (2) develop morphologically identical ookinetes and oocysts in mosquitoes (Fig. 9 e, g) and (3) have the same mitochondrial genetic lineages.

Control dissected mosquitoes remained uninfected throughout the course of the experiment. We revealed partial sporogonic development of *H. tartakovskyi* and *H. balmorali* in the head, thorax and midgut of experimental *O. cantans*. Ookinetes and/or oocysts were reported in 100 % of infected and dissected insects. Both parasite species developed approximately synchronously in this mosquito. Development of sporozoites was not observed in oocysts. Sporozoites were not seen in the salivary glands, indicating abortive sporogonic development of both parasite species at the oocyst stage.

Microscopic examination of preparations of the midgut content and the whole

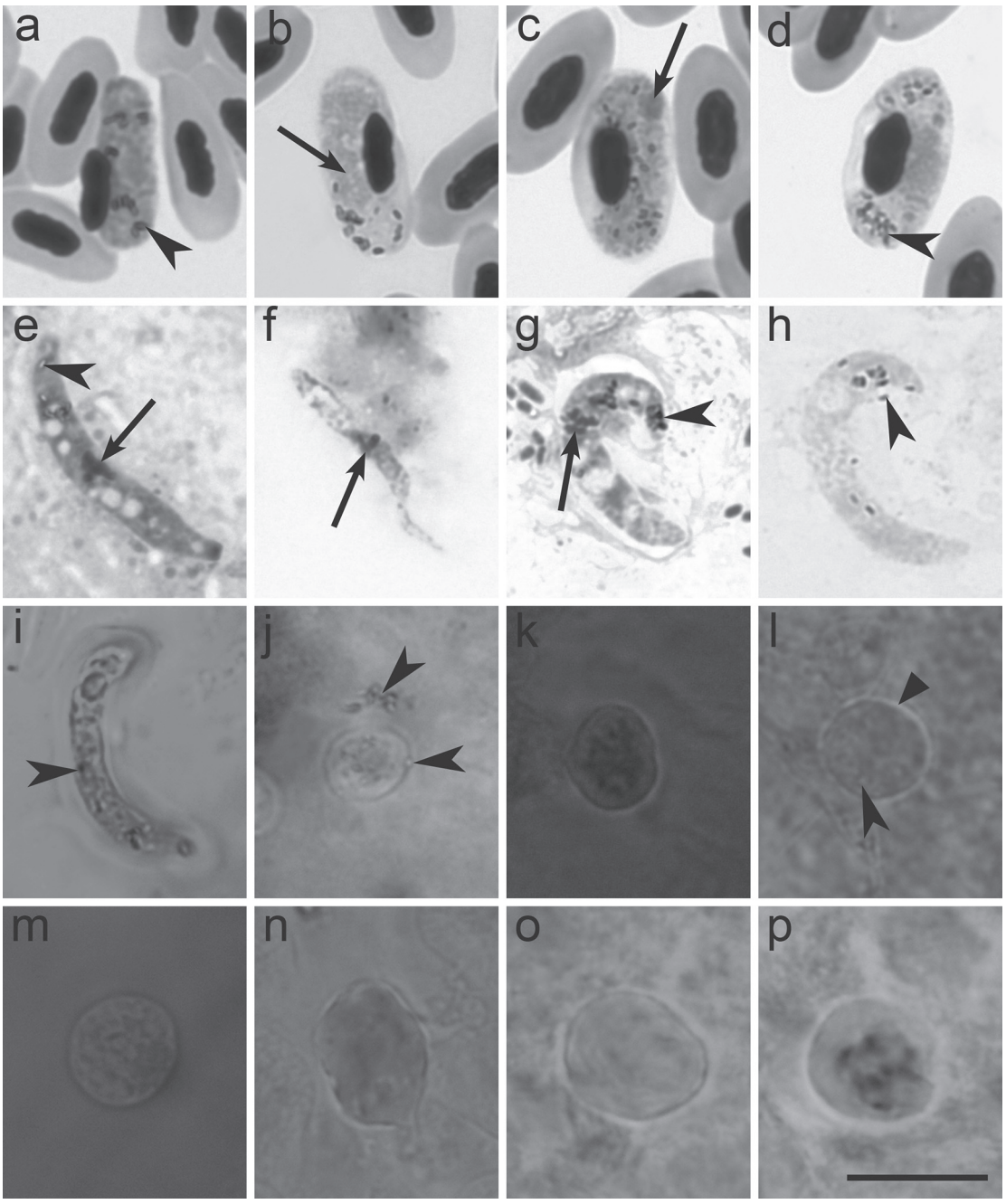

Fig 9. Gametocytes and sporogonic development of *Haemoproteus tartakovskyi* (a, b, e, f, i – k, n – p) and *Haemoproteus balmorali* (c, d, g, h, l, m) in experimentally infected mosquito *Ochlerotatus cantans*. a – d Mature macrogametocytes (a, c) and microgametocytes (b, d) in the blood of donor birds before infection of mosquitoes. Ookinetes e – i in the midgut content (e – h) and on surface of the midgut wall (i): mature ookinetes 24 h post – infection (e) and 48 h post – infection (g), i mature ookinete 5 days post – infection (dpi), degenerating ookinetes 5 dpi (f) and 4 dpi (h). Oocysts j – p on surface of the midgut wall: j, l growing oocysts 5 dpi; k, m fully grown oocysts 8 dpi (k) and 12 dpi (m); n – p degenerating oocysts 11 dpi (n) and 16 dpi (o, p). Note presence of black (melanised) areas in degenerating oocysts (n – p). a – h Giemsa stained thin films. i – p Formalin fixed and Erlich's hematoxylin – stained whole mounts of midguts. Long arrows nuclei of parasites. Simple arrowheads pigment granules. Triangle arrowhead capsular – like wall of oocysts. Bar = 10 μm

mounts of midgut revealed the following features. Numerous mature ookinetes of both parasites were seen in the midgut content between 1 and 3 dpi (Fig. 9 e, g); their number decreased markedly between 4 and 5 dpi when a few degenerating ookinetes were observed (Fig. 9 f, h). The degenerating ookinetes possessed the pale - stained cytoplasm and poorly visible or even invisible nuclei; vacuole-like spaces were invisible or poorly visible in the degenerating parasites (compare Fig. 9 e, g with Fig. 9 f, h). Ookinetes developed markedly asynchronously: They were seen on surface of the midgut wall in the whole mounts preparations until 5 dpi (Fig. 9 i). Numerous roundish oocysts of *H. tartakovskyi* (Fig. 9 j, k, n – p) and *H. balmorali* (Fig. 9 l, m) were observed on surface of the midgut wall between 4 and 16 dpi.

Oocysts developed markedly asynchronously, resulting in the presence of parasites of different sizes in all preparations. Flattened oocysts specimens of *H. tartakovskyi* (n = 21) measured 6.7 – 11.4 (on average, 8.7 ± 1.4 μm) in their largest diameter on 6 dpi; the parasites measures 6.6 – 13.7 (on average of 9.2 ± 2.1 μm) on 9 dpi, and then persisted morphologically unchanged until 11 dpi when degeneration features, such as the constriction of their form (Fig. 9 n – p) and the melanisation (Fig. 9 p), gradually develop. Pigment was evident in oocysts (Fig. 9 j), but no pattern was discernible. Residual pigment granules (remnants of ookinete residual bodies) are readily visible in the midgut (Fig. 9 j). Only degenerating oocysts were seen in midgut wall between 15 and 17 dpi. There was no significant difference discernible between *H. tartakovskyi* and *H. balmorali* in patterns of development, morphology, or size of oocysts.

Microscopic examination of histological preparations of the head, thorax and abdomen revealed the following features. Numerous red blood cells, including the infected ones, were visible in sections of gut located in the head, thorax and abdomen of infected mosquitoes on 1, 2 and 3 dpi (Fig. 10 a, b). Numerous ookinetes of *H. tartakovskyi* and *H. balmorali* were seen in the same sections (Fig. 10 a, b), indicating successful sexual process and initial sporogonic development in these parts of the body soon after the infected blood meal. Apparently, ookinetes find no barrier between the gut contents and the gut wall or haemocoele, as numerous ookinetes were present in the head, thorax and abdomen tissues (Fig. 10 c – f). In transverse sections, the ookinetes looked like roundish or oval bodies, often with adjacent pigment granules; the parasites looked the same in histological sections of the abdomen, thorax and head (Fig. 10 a – f). Oocysts of both parasite species (Fig. 10 g – l) were observed in histological sections of the

head, thorax and abdomen between 4 and 15 dpi, indicating active movement of ookinetes and initial sporogony in these body parts. Development of sporozoites was not seen in oocysts.

PCR and sequencing revealed natural *Haemoproteus* infections in one of 40 (2.5 %) pools of wild - caught non - fed *O. cantans*, indicating that this mosquito naturally takes blood meals on infected birds at the study site. The lineage hDELURB11 of *Haemoproteus hirundinis* was detected in the naturally infected pool. We consider infections in experimental mosquitoes to have been experimentally induced because (1) haemosporidian parasites were not reported in control mosquitoes, (2) only the lineages hSISKIN1 and hROBIN1 were present

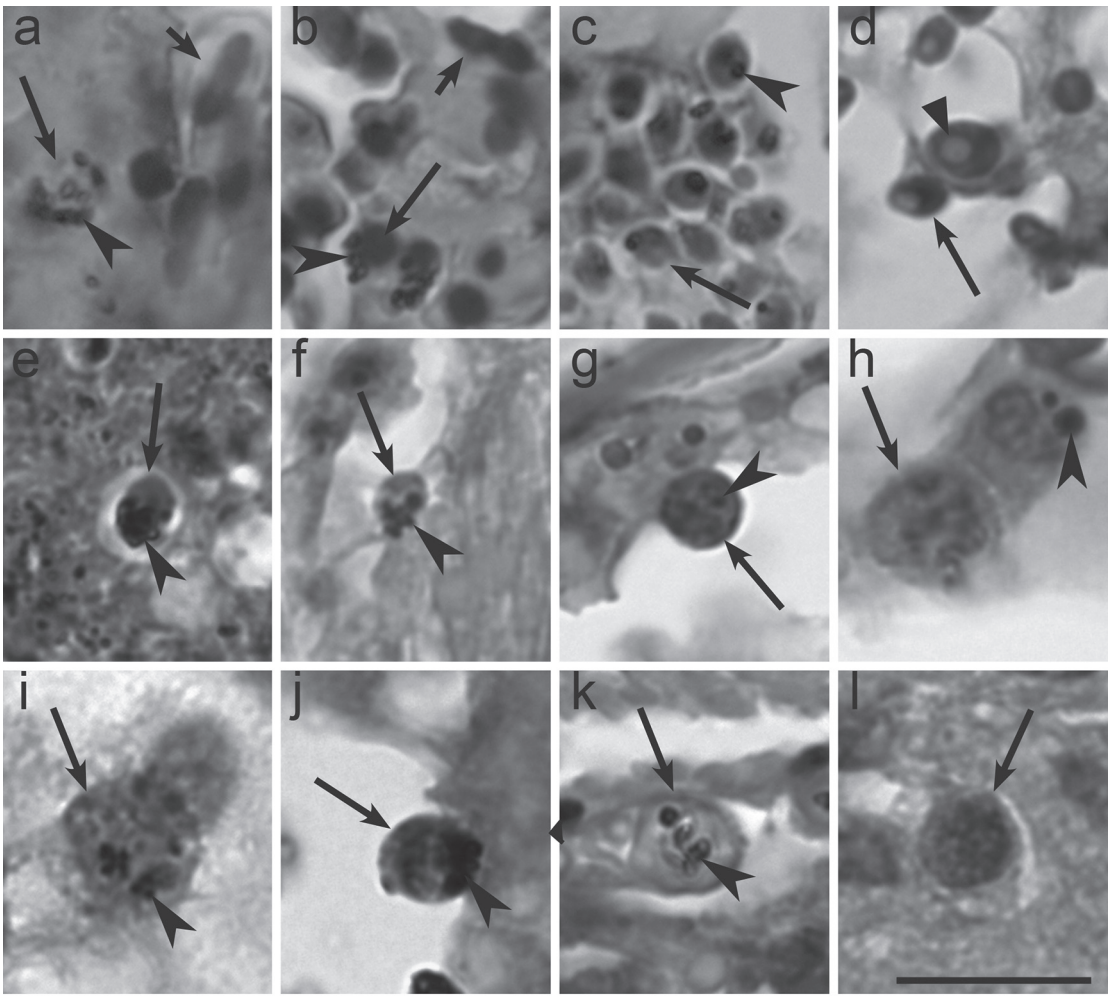

Fig. 10. Sporogonic stages *of Haemoproteus tartakovskyi* (a – c, e, f, i, j, l) and *Haemoproteus balmorali* (d, g, h, k) in histological sections of experimentally infected mosquito *Ochlerotatus cantans*; the parasites look like roundish bodies in the transverse sections. Ookinetes in the head (a, f), thorax (b, d) and midgut (c) on 1 day post-infection (dpi, a – c), 2 dpi (d, e) and 3 dpi (f); note presence of red blood cells in the head (a) and thorax (b), and of vacuole – like spaces in ookinetes of *H. balmorali* (d). Oocysts in the thorax (g – i, k, l) and midgut (j) on 6 dpi (g – j) and 15 dpi (k, l). Note presence of black (melanised) areas in the degenerating oocyst (l). Formalin fixed and hematoxylin - eosin stained histological sections. Long arrows parasites. Short arrows red blood cells. Simple arrowheads pigment granules. Triangle arrowhead vacuole – like space. Bar= 10 μm.

in experimental insects, (3) numerous ookinetes of *H. tartakovskyi* (Fig. 9 e) and *H. balmorali* (Fig. 9 g) were observed in each infected mosquito examined microscopically and (4) 100 % and approximately 50 % (Table 4) of the experimental mosquitoes were positive by microscopic examination and PCR - based methods for experimental parasites, respectively.

The results of the PCR assays from experimentally infected mosquitoes are summarised in Table 4. Only half of the infected insects were PCR positive. In both infections, the head, thorax and/or abdomen were PCR positive until 15 dpi. No positive PCR signals were reported in these parts of the body 16 and 17 dpi, except one case in *H. balmorali* infection on 17 dpi. The detection of parasites (Table 4) was significantly less sensitive from the head than from the thorax (for both infections, $\chi 2 > 7.3$, $P < 0.01$) and the abdomen (for both infections, $\chi 2 > 6.3$, $P < 0.05$). Parasites were approximately equally detectable in the thorax and abdomen (for both infections, $\chi 2 < 0.1$, $P > 0.8$). The stage of blood digestion in abdomen changed from fresh (fully fed mosquitoes) to completely digested to the end of our study. As sporozoites did not develop in oocysts, the finding of PCR - positive results from head, thorax and abdomen of mosquitoes, in which parasite development was abortive, shows that PCR positivity (Table 4)

Table 4.
Time cause of polymerase chain reaction (PCR) - based detection of *Haemoproteus* infection from mosquitoes *Ochlerotatus cantans* fed on infected blood meal (*H* head, *T* thorax, *A* abdomen).

Day post infection	*H. tartakovskyi* (lineage hSISKIN1)					*H. balmorali* (lineage hROBIN1)				
	Number of infected mosquitoes	Number of PCR positive				Number of infected mosquitoes	Number of PCR positive			
		Mosquitoes	H	T	A		Mosquitoes	H	T	A
1	4	3	0	3	3	4	2	0	2	2
2	3	3	0	1	3	4	4	0	3	4
3	4	3	0	3	3	3	2	0	2	2
4	4	2	2	2	2	3	2	0	1	2
5	4	3	1	2	3	1	1	0	1	1
6	3	2	2	2	2	2	1	1	0	0
8	3	1	1	1	1	2	1	0	1	1
9	2	1	0	0	1	2	2	1	1	2
10	4	2	0	2	1	3	0	0	0	0
11	2	1	0	1	0	3	1	1	1	1
12	2	1	0	1	0	2	1	0	1	0
14	2	1	0	1	0	2	1	1	1	0
15	5	3	1	3	1	3	2	1	2	1
16	3	0	0	0	0	3	0	0	0	0
17	4	0	0	0	0	5	1	0	1	0
Total	49	26 (53.1) [a]	7 (14.3)	22 (44.9)	20 (40.8)	42	21 (50.0)	5 (11.9)	17 (40.5)	16 (38.1)

[a] Percentage is given in parentheses.

does not necessarily indicate a potential vector species. Bird DNA was detectable in the head, thorax and abdomen of mosquitoes between 1 and 4 dpi; it was not detectable later, except 1 case in an abdomen 5 dpi. These data correspond to the visual observation of red blood cells in histological sections of these parts of the mosquito body (Fig. 10 a, b).

3.4.2. Discussion

The key results of this experimental study are that two *Haemoproteus* (*Parahaemoproteus*) species (1) undergo sexual processes and produce ookinetes throughout the entire digestive tract of engorged mosquitoes, including the head, thorax and abdomen; (2) ookinetes find no barrier in their way from gut contents to the gut wall and haemocoele, resulting in the presence of parasites throughout the body of the mosquitoes; (3) develop oocysts in the head, thorax and midgut wall of mosquitoes; and (4) abort sporogonic development at the oocyst stage without formation of sporozoites. Importantly, *Haemoproteus* spp. DNA is detectable in the head, thorax and abdomen of infected mosquitoes for several weeks, indicating relatively long - time persistence of *Haemoproteus* parasites in resistant insects following an infected blood meal. In spite of abortive sporogonic development, mosquitoes have been scored as infected by PCR (Table 4) when, in fact, they are not competent vectors of the parasites. This makes obstacles in direct application of PCRbased detection methods in vector research, particularly in wildlife.

It is sometimes deemed that amplification of parasite DNA in the thorax of non engorged mosquitoes and other haemosporidian vectors suggests the presence of sporozoites and might indicate involvement in transmission (Ishtiaq et al., 2008; Kimura et al., 2010; Njabo et al., 2011; Kim and Tsuda, 2012; Ventim et al., 2012). The present study shows that this can be incorrect; that is, not only because of possible presence of remnants of infected red blood cells in the thorax (Kim et al., 2009; see Fig. 10 b) but also due to presence of ookinetes and oocysts (Fig. 10 b, d, g – i, k, l), which develop and persist in resistant insects for some time. Interestingly, Kim et al. (2009) investigated sporogonic development of *Plasmodium gallinaceum* in resistant experimentally infected mosquito *Culex pipiens pallens*; abortive parasite development was detectable by PCR – based methods up to 13 dpi.

It is important to note that histological sections of the head and thorax of fully engorged mosquitoes were overfilled with ookinetes on 1 dpi (Fig. 10 a,

b). That can be explained by rather rapid gametogenesis and ookinete development in haemosporidian parasites (Garnham, 1966; Sinden, 1998; Valkiūnas, 2005). In many species of avian *Haemoproteus*, the sexual process can be readily induced in vitro using simple media containing only infected blood and anticoagulant (3.7 % solution of sodium citrate): gametogenesis terminates within several minutes and mature ookinetes develop within 12 hr post infection of mature gametocytes to air at 20 ± 1° C (Valkiūnas et al., 2013b). Such stimuli are unnecessary in avian *Haemoproteus* parasites. Because numerous infected red blood cells were observed in the digestive tract located in the head (foregut) and thorax (thoracic midgut) areas of engorged mosquitoes between 1 and 3 dpi, it is unsurprising that we found ookinetes developing in these parts of the body (Fig. 10 a, b, d, f), but this has not previously been documented in haemosporidians. This study shows that traditional opinion about the haemosporidian ookinete development and initial sporogony mainly in the abdominal midgut of dipteran insects requires partial reconsideration. Further studies are needed for better understanding the fate of such parasites in the life cycle of other haemosporidians.

This study shows that results of such studies, should be interpreted with caution because PCR will amplify haemosporidian DNA regardless of the life stages present in blood - sucking insects; it cannot distinguish between infective (sporozoite) and non - infective (ookinete, oocyst) sporogonic stages (Table 4). Hence, PCR detection of haemosporidian genetic material in blood-sucking insects, even several weeks after an infected blood meal, does not necessarily mean that this material originated from parasites that are capable of producing viable sporozoites, the essential stage for haemosporidian transmission. The present study shows that the same is true for *Haemoproteus* species, indicating that the former reports of lineages of *Haemoproteus* spp. in mosquitoes (Ishtiaq et al., 2008; Njabo et al., 2009; 2011; Glaizot et al., 2012) and avian *Plasmodium* spp. in biting midges (Martínez-de la Puente et al., 2011), which are not known to be competent vectors of these parasites, are likely to represent PCR - based detection of abortive parasite development. Microscopic examination was approximately twice as sensitive as PCR in the detection of parasites during this study. However, the PCR - based detection was less time consuming. Additionally, the applicability of insect dissection and microscopic examination methods is limited in wildlife due to predominant light infections and low parasite prevalence (often < 1 %) in the majority of vector populations (Garnham, 1966; Beier, 1998;

Valkiūnas, 2005). Both tools complement each other and are worth combining in vector research. It should be mentioned that the lower number of positive molecular detections of *Haemoproteus* infections from the head of mosquitoes (Table 4) was to be expected, due to inhibitory effect of the hard exoskeleton on PCRs (Arez et al., 2000; Kim et al., 2009).

Numerous species of blood - sucking dipteran insects usually are present at each study site; it is difficult to test them for involvement in haemosporidian parasite transmission using microscopic examination. Therefore, it is hard to imagine future ecological and epidemiological vector studies of wildlife haemosporidians without application of fast PCR - based detection of infections. Two steps can be recommended in haemosporidian vector research at a study site in wildlife. First, the PCR - based detection methods are worth applying in testing blood-sucking insects for the presence of haemosporidian genetic material. This would markedly accelerate search of most probable vector species by determining significant links between blood - sucking insects on the one hand and haemosporidian species on the other hand. Recent studies by Malmqvist et al. (2004), Ishtiaq et al. (2008), Kimura et al. (2010), Carlson et al. (2011), Ejiri et al. (2011), Martínez-de la Puente et al. (2011), Glaizot et al. (2012), Kim and Tsuda (2012) and Ventim et al. (2012) are examples of such research. However, the significant molecular links between parasite haplotypes and blood - sucking insects could only indicate insect - parasite relationships, but do not prove involvement of the insects in transmission. Second, the report of sporozoites in bloodsucking insect fed on infected hosts is an essential next step for the definitive demonstration that these insects could act as vectors (Njabo et al., 2009).

It is important to note that some vectors are incapable of transmitting infection by bite even if sporozoites develop and present in salivary glands (Garnham, 1966; Nayar et al., 1980; Valkiūnas, 2005; Santiago-Alarcon et al., 2012). Ideally, experimental research is needed to demonstrate viability of salivary gland sporozoites. Because of the complicated life cycles of haemosporidians, microscopic approaches and experimental research remain essential and should be applied in parallel with PCR - based detection tool in vector studies, particularly in wildlife. It is worth mentioning that PCR - based methods provide straightforward opportunities for investigation of feeding specialisation of blood-sucking insects and other arthropods; it is easy to detect and identify DNA of bloodsource animals in engorged insects (Malmqvist et al., 2004; Ejiri et al., 2011; Kim and Tsuda, 2012). However, the opportunities to use the host DNA markers are limited in such re-

search because of rapid DNA degeneration in the digestive tract of insects. Usually, host DNA can be amplified only up to 3 – 4 days after a blood meal. Because haemosporidian parasites can survive for several weeks both in vectors (Garnham, 1966; Valkiūnas, 2005) and resistant insects (Kim et al., 2009; this study), we recommend using haemosporidian parasite molecular makers in determining possible links between blood - sucking insects and their blood - source animals (see above). Many haemosporidian lineages are restricted to a few host species (Bensch et al., 2009); reports of such lineages indicate possible insect–vertebrate host associations. For example, our finding in this study of *H. hirundinis* (hDELURB11) in *O. cantans* indicates that *O.cantans* naturally takes blood meals on the house martin *Delichon urbica*, a specific avian host of this parasite.

3.5. Experiment 5. Determination of virulence of *Haemoproteus* parasites for bird – biting mosquitoes and patterns of their development in these insects

3.5.1. Results

Control mosquitoes remained uninfected throughout the course of the experiment, as determined both by microscopic examination and PCR – based testing. Single infections were recorded in all donor birds, as confirmed both by PCR – based detection and microscopic examination of blood films. *H. balmorali* (lineage hCOLL3, host was one pied flycatcher), *H. lanii* (lineage hRB1, host was one red-backed shrike, and lineage hRBS2, host was one redbacked shrike) and *H. tartakovskyi* (lineage hSISKIN1, hosts were three siskins) were identified both by morphological characters (Fig. 11 a – c) and sequence data. The same parasite lineages were detected in all experimentally infected mosquitoes fed on corresponding bird species.

Genetic difference between the lineages hRB1 and hRBS2 of *H. lanii* was negligible (0.4 %); it was greater (4.8 – 6.4 %) among other reported *Haemoproteus* spp. lineages. For each experiment, intensity of parasitaemia in donor birds is indicated in Fig. 12.

Single isolates of *H. balmorali* and *H. lanii* were used in each experiment. Three isolates from different individual siskins were assumed to be biologically identical because isolates (1) have the same mitochondrial haplotypes, (2) show morphologically indistinguishable gametocytes in the peripheral blood (Fig. 11 c) and (3) produce morphologically identical ookinetes in mosquitoes (Fig. 11 f).

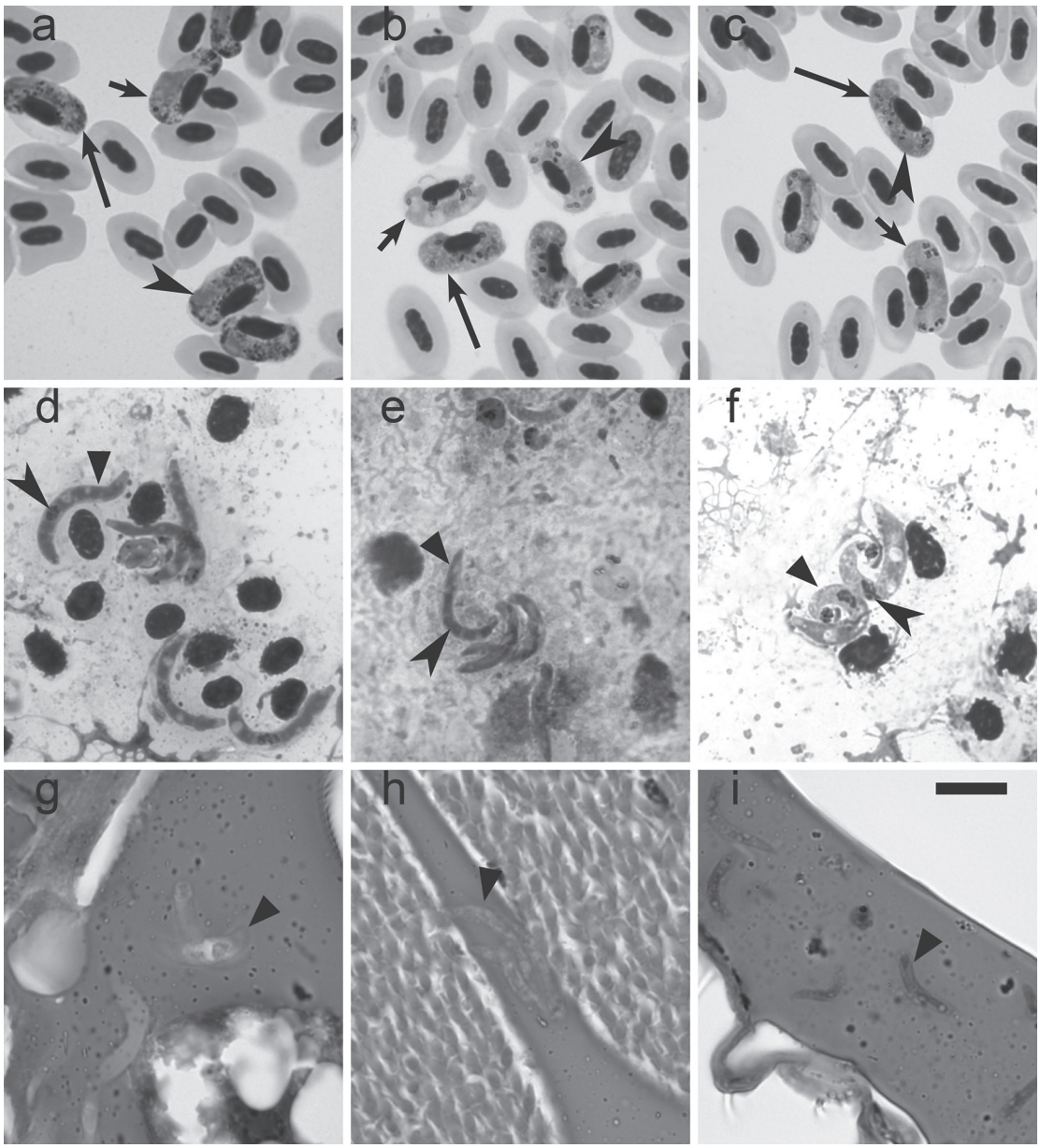

Fig 11. *H. balmorali* (a, d), *H. lanii* (b, e, g – i) and *H. tartakovskyi* (c, f) in the blood of donor birds (a – c), midgut preparations (d – f) and histological sections (g–i) of experimentally infected mosquitoes *O. cantans*. a – c Mature gametocytes in the peripheral blood of birds before infection of mosquitoes. Ookinetes in the midgut content (d – f) and histological sections (g – i) in the head (g), thorax (h) and abdomen (i) 24 h postexposure of mosquitoes. a – f Giemsa - stained thin films. g – i Formalin - fixed and haematoxylin - eosin stained histological sections. Long arrows – macrogametocytes, short arrows microgametocytes, simple arrowheads parasite nuclei, and triangle arrowhead ookinetes. Bar =10 μm

In wild – caught non – fed *O. cantans*, PCR detected *P. relictum* (lineage pSGS1) infection in one pool of mosquitoes (1.5 % of pools were PCR positive), indicating that *O. ca*ntans naturally feeds on birds at the study site. We consider infections in exposed wild - caught mosquitoes to have been experimentally induced because (1) only *Haemoproteus* spp. lineages present in donor birds were recorded in corresponding groups of exposed insects; (2) numerous ookinetes of *H. balmorali* (Fig. 11 d), *H. lanii* (Fig. 11 e) and *H. tartakovskyi* (Fig. 11 f) were

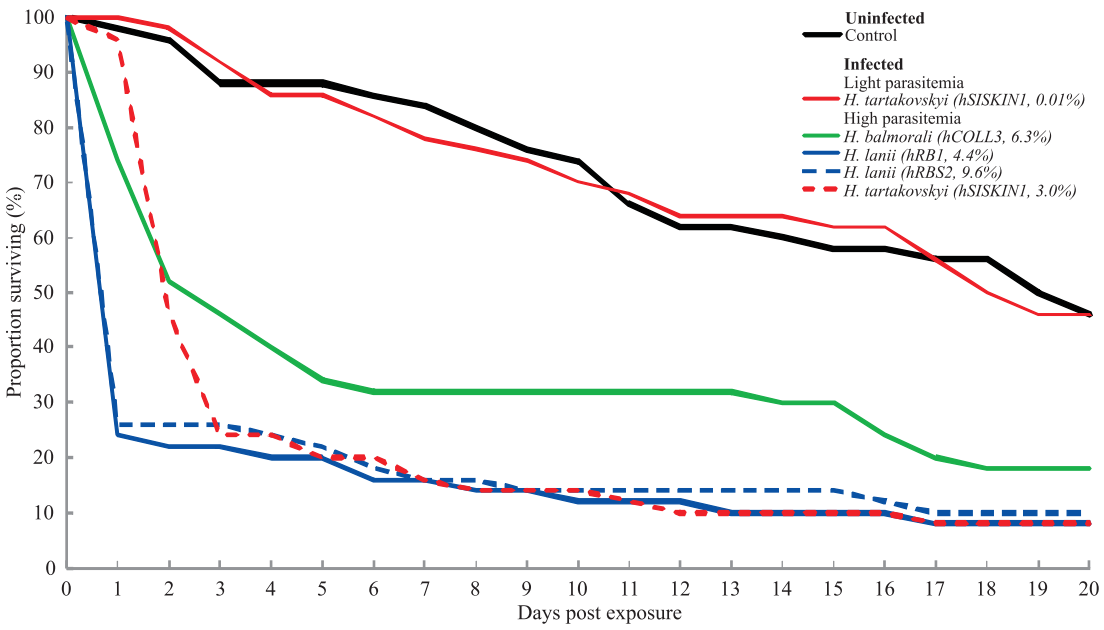

Fig 12. Cumulative percentage of survival in uninfected (control) and *Haemoproteus* spp.-infected groups of *O. cantans*. In each group, 50 mosquitoes were exposed.

observed in each corresponding group of infected mosquito examined microscopically; and (3) 100 % of dissected experimental mosquitoes were positive by microscopic examination and PCR - based methods for corresponding experimental parasites.

For all groups of infected mosquitoes, microscopic examination revealed numerous ookinetes in the midgut contents of all insects dissected between 0.5 and 4 dpi. The parasites overfilled mosquito midguts in all groups fed on heavily infected blood meals (Fig. 11 d – f), but a few ookinetes were seen in mosquitoes exposed to lightly infected blood meal.

Examination of histological sections of mosquito midguts showed the same results. Additionally, numerous ookinetes were seen in histological section of the digestive tract and adjacent tissues located in the head (Fig. 11 g), thorax (Fig. 11 h) and abdomen (Fig. 11 i) as soon as 1 dpi. Ookinetes find no barrier in their way from gut contents to the gut wall and haemocoel, resulting in the presence of the parasites throughout the body 1 and 2 dpi.

Migrating ookinetes overfill haemocoel and damage tissues in the head, thorax and abdomen of infected mosquitoes (Fig. 11 g – i). Survival rate of exposed mosquitoes cumulative data on survival rates of uninfected (control) and experimentally infected mosquitoes are shown in Fig. 12. No difference was discernible in the survival rate of control mosquitoes and the experimental group fed on blood meal with light parasitaemia of *H. tartakovskyi* (P = 0.48). In other words,

light *Haemoproteus* infection is relatively benign to mosquitoes. However, the survival rates of both these groups were significantly higher than the survival of four groups fed on meals with high parasitaemia (in comparison to control, $P < 0.001$ for all these groups), testifying to the marked virulence of heavy *Haemoproteus* infections to mosquitoes (Fig. 12).

The survival rates of mosquito groups exposed to high parasitaemia of the lineages hRB1 and hRBS2 of *H. lanii* did not differ significantly from each other ($P = 0.42$), but the survival of both these groups was significantly lower than that of groups fed on meals with high parasitaemia of *H. tartakovskyi* ($P < 0.01$) and *H. balmorali* ($P < 0.001$) (Fig. 12). The survival rate of mosquito groups exposed to high parasitaemia of *H. tartakovskyi* and *H. balmorali* did not differ significantly from each other ($P = 0.09$). In all mosquito groups fed on meals with high parasitaemia, the survival rates sharply decreased between 1 and 3 dpi (Fig. 12). The survival rates decreased amazingly rapidly in both groups fed on blood meals with high parasitaemia of *H. lanii* (lineages hRB1 and hRBS2).

It is interesting to note that the intensity of parasitaemia in the meal infected with the hRBS2 lineage was twofold higher than that in the meal infected with hRB1, but the survival rates of both groups decreased synchronously. In both these groups, approximately 60 % of insect survived 0.5 dpi, but all mosquitoes were alive in groups exposed to *H. balmorali* and *H. tartakovskyi* infections at the same time ($P < 0.001$ for both these groups in comparison to both groups infected with *H. lanii*). For 4 dpi, the percentage of survived control mosquitoes (88 %) was 2.2-, 3.6- and 4.4 – fold greater than that of groups fed on meals with high parasitaemia of *H. balmorali*, *H. tartakovskyi* and *H. lanii*, respectively ($P < 0.001$ for each group in comparison to control). Survival rate gradually slightly decreased in all mosquito groups, including control, from 4 to 5 dpi to the end of this study (Fig. 12). On 20 dpi, the same number of mosquitoes (46 %) survived both in the control group and the group of mosquitoes fed on meal with light parasitaemia, but the survival rate of experimental mosquitoes fed on heavily infected meals was between 2.6- and 5.8- fold lower ($P < 0.001$ for each group in comparison to control).

3.5.2. Discussion

Numerous experimental studies address detrimental effects of these parasites on mosquito survival (Ferguson and Read, 2002; Lehane, 2005), but knowledge about virulence of widespread *Haemoproteus* infections to mosquitoes is insuf-

ficient (Valkiūnas et al., 2013c). This is unfortunate because haemoproteids are present in the majority of terrestrial bird populations, often at high infection prevalence, reaching 30 – 100 % in many bird species (Greiner et al., 1975; McClure et al., 1978; Peirce, 1981; Merino et al., 2000; Valkiūnas, 2005; Zehtindjiev et al., 2013). These parasites might damage bird - biting mosquitoes and contribute to their mortality; however, there is no experimental evidence about that.

This study is in accord with the former experimental observation (Valkiūnas et al., 2013c), indicating that avian haemoproteids undergo initial sporogony and produce numerous ookinetes, which migrate throughout the entire body of *O. cantans*. This mosquito cannot transmit haemoproteids because the sporogonic development is abortive (ectopic).

The key result of this study is that widespread *Haemoproteus* (*Parahaemoproteus*) species are markedly virulent and rapidly kill the majority of infected *O. cantans* mosquitoes, which are abundant at our study site and willingly take blood meals on birds (Bernotienė, 2012). It is likely that the simple physical damage of mosquito tissues by ookinetes in the head, thorax and abdomen is the main reason of the sharply reduced survival rate between 0.5 and 2 dpi (Fig. 12). During this period of time, numerous ookinetes develop and migrate throughout the body of infected insects, but oocysts are still absent (Valkiūnas et al., 2013c). Physical damage due to perforation of tissues should be directly related to the ookinete numbers, which depend on the intensity of gametocytaemia in donor birds. This explains why the mosquito survival rate markedly depends on the intensity of infection (Fig. 12).

It is important to note that the survival rates of two mosquito groups infected with genetically similar lineages of *H. lanii* were the same, but the intensity of parasitaemia was approximately twofold higher (4.4 and 9.6 %) in blood meal of one of the groups. However, the same number of insects (25 %) survived in both these groups 1 dpi (Fig. 11). These data indicate a possible existence of a threshold level of parasitaemia, which leads to the development of numerous ookinetes, which cause a dramatic mosquito damage resulting in maximum possible mortality in a group; further increase of parasitaemia might not influence significantly the insects' mortality, which reaches a maximum at lower (threshold) parasitaemia. In *H. lanii* infection, a parasitaemia close to 4 % might be the threshold level.

It is worth mentioning that the rapid decrease of survival in two groups infected with *H. lanii* between 0.5 and 1 dpi can be hardly explained only by high

parasitaemia in their blood meals (4.4 and 9.6 %); the mosquito group exposed to *H. balmorali* infection also was fed on meal with high parasitaemia (6.3 %), but the majority of insects survived in this group 1 dpi (Fig. 12). It is probable that the rate of development of mature ookinetes, which are capable of moving via tissues, can be an important factor influencing a more rapid decrease of survival in two groups infected with *H. lanii* in comparison to groups infected with *H. tartakovskyi* and *H. balmorali*. Under the same in vitro conditions, mature ookinetes of *H. lanii* develop within 3 hr, but ookinetes of *H. tartakovskyi* and *H. balmorali* develop several - fold slower (Valkiūnas, 2005). Because only mature ookinetes are capable of migration, this can explain marked mortality in both *H. lanii* infected groups, but lack of mortality in both *H. tartakovskyi*- and *H. balmorali* -infected groups 0.5 dpi.Tissue perforation by *Plasmodium* sp. ookinetes was reported to increase susceptibility of mosquitoes to bacterial infection and other parasites during subsequent feeding (Lehane, 2005). However, this is unlikely a case in our experiments because numerous mosquitoes died too rapidly after exposure, i. e., many insects exposed to *H. lanii* infection were dead 0.5 dpi; this time is hardly enough for the development of prominent secondary bacterial infection. For the same reason, it is hardly possible that such factors as (1) the immunity suppression to re - infections, (2) the physiological disruption and (3) the behaviour modifications can be attributed to mechanisms causing damage of *O. cantans*, as is a case in some long - lasting *Plasmodium* sporogonies in susceptible mosquito species (Beier, 1998; Jahan et al., 1999; Barillas-Mury et al., 2000; Lehane, 2005).

This study shows that *Haemoproteus* infections are more virulent to mosquitoes than *Plasmodium* infections, in which severe mortality was not reported within 1 dpi (Ferguson and Read, 2002; Lehane, 2005). This can be explained by differences in these haemosporidian parasites during initial sporogony. In species of avian *Haemoproteus*, the sexual process and development of ookinetes can be readily induced in vitro using simple media containing only infected blood and anticoagulants; gametogenesis terminates within several minutes, and mature ookinetes of many parasite species develop within several hours after exposure of mature gametocytes to air (Valkiūnas, 2005). *Plasmodium* parasites do not initiate sexual process and development of ookinetes in vitro at such simple conditions; they require the presence of vector - derived xanthurenic acid and blood - derived factors (Sinden, 1998; Arai et al., 2001). Such stimuli are unnecessary in avian *Haemoproteus* parasites, resulting in sexual process and

ookinete development throughout the entire digestive tract of fully engorged mosquitoes, including the head (foregut) and thorax (thoracic midgut) soon after the exposure (Valkiūnas et al., 2013c). This is not a case in *Plasmodium* parasites (Garnham, 1966; Sinden, 1998). Because *Haemoproteus* ookinetes find no barrier in their way from gut contents to the adjacent tissues of the head and thorax, these parasites cause marked physical damage throughout the body of the mosquitoes. That contributes to high virulence of *Haemoproteus* parasites in mosquitoes, as compared to *Plasmodium* spp. High *Haemoproteus* parasitaemia (> 1 %) has been often reported in many species of avian hosts, particularly during bird breeding season, which usually coincides both with an active transmission of haemosporidian parasites and a high abundance of blood-sucking insects (Garnham, 1966; Valkiūnas, 2005). Due to high infection prevalence and intensity in birds, this period of time should be dangerous to bird-biting mosquitoes. However, there is no information about effects of *Haemoproteus* parasites on mosquito populations.

A few studies address effects of wildlife haemosporidian parasites on survival of blood - sucking insects. It has been shown that the survival rates of (1) *C. impunctatus* infected with avian *Haemoproteus* spp. and (2) *Simulium* spp. infected with *Leucocytozoon* spp. were inversely proportional to the intensity of parasitaemia in birds they had fed on (Desser and Yang, 1973; Allison et al., 1978; Valkiūnas et al., 2013c). These are examples of mortality caused in the natural (co - evolved) vector - parasite combinations, in which sporogony is completed, and sporozoites develop in survived insects. This communication is based on the unnatural (not co - evolved) vector - parasite associations, but the results were similar - the survival rate of mosquitoes is significantly reduced when feeding upon birds with high gametocytaemia. It seems that heavy *Haemoproteus* infections are virulent both in co - evolved and not co - evolved vector - parasite associations. In nature, individuals are exposed to numerous environmental factors; thus,mosquito mortality due to *Haemoproteus* parasites may be even more pronounced than was recorded in the laboratory (Fig. 12). Reduced survival of blood-sucking insects implies a possible reduced involvement in transmission of other infections by decreasing the number of survived biting females. This might be significant epidemiologically but remains insufficiently investigated. Further studies are needed to understand fully consequences of pathogenesis of *Haemoproteus* infections on mosquitoes and other blood - sucking insects, particularly during abortive sporogonic development, which remain insufficiently investigated.

3.6. Experiment 6. Determinatioon of the development of *Plasmodium homocircumflexum* (lineage pCOLL4) in the mosquitoes *Culex pipiens pipiens, Culex pipiens pipiens* form *molestus* and *Aedes vexans*

3.6.1. Results

We observed the development of ookinetes of *Plasmodium homocircumflexum* in the midguts of *C. p. pipiens* (Fig. 13 a, d), *C. p. pipiens* f. *molestus* (Fig. 13 b, e)

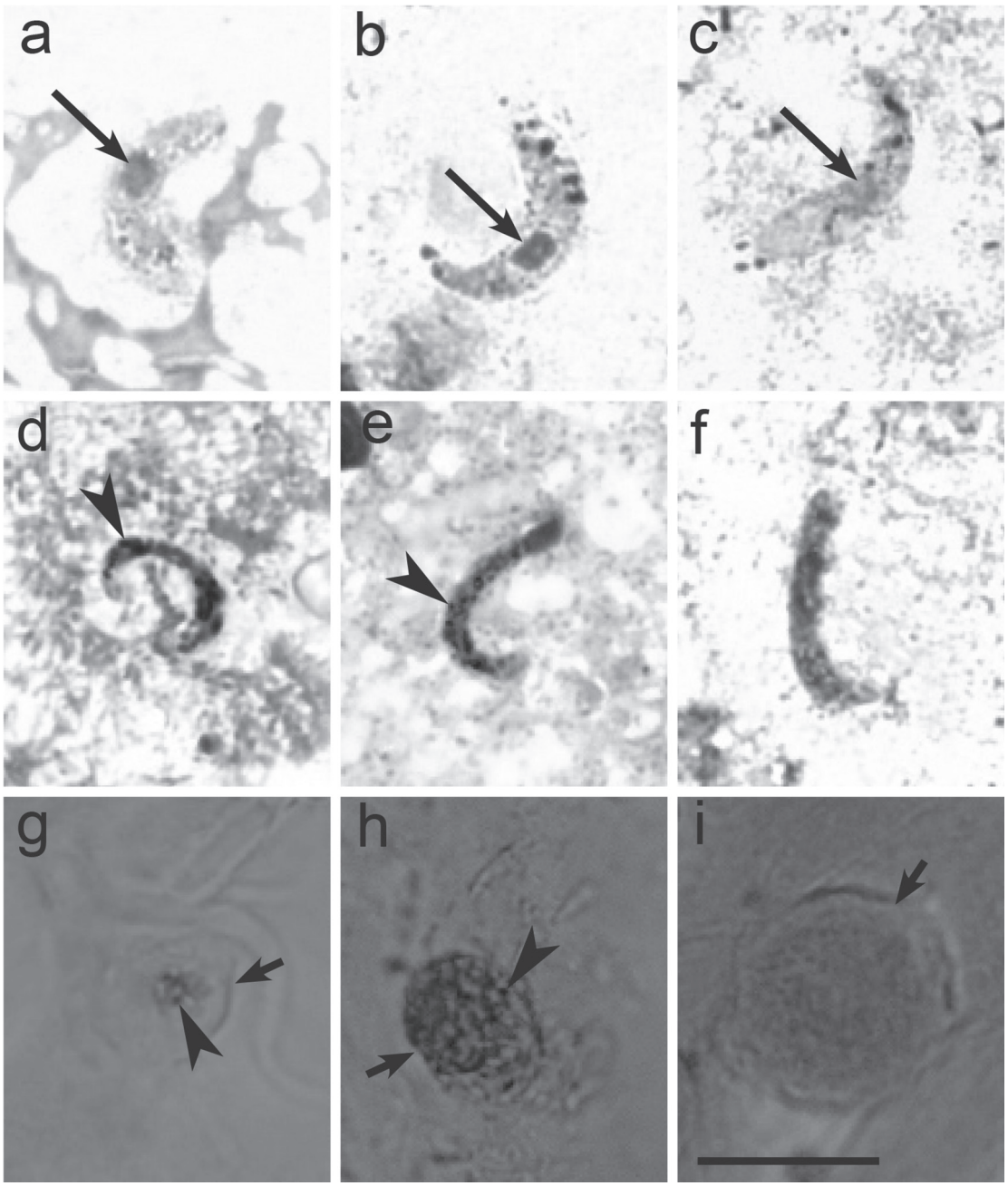

Fig 13. Sporogonic stages of *Plasmodium homocircumflecum* (lineage pCOLL4) from the midgut of *Culex pipiens pipiens* (a, d, g), *Culex pipiens pipiens* form *molestus* (b, e, h) and *Aedes vexans* (c, f, i). a – c – ookinetes; d – f – degraded ookinetes; g – i – degraded oocysts. Long arrows – nuclei of parasites. Short arrows – oocysts. Arrowheads – pigment granules. Giemsa - stained thin blood films (a – f). Midgut whole mounts stained with Erlich's hematoxylin (g – i). Scale bar = 10 μm.

24 hr pi, and also in *Aedes vexans* 48 hr pi (Fig. 13 c, f). Ookinetes were seen in 100 % of infected mosquitoes of all species. Ookinetes were elongated worm - like bodies, which possessed slightly of centre located nuclei, large "vacuoles" and pigment granules (Fig. 13 a – c). The oocysts were observed 6 – 20 dpi in *C. p. pipiens,* 10 – 20 dpi in *C. p.* f. *molestus* and 9 – 14 dpi in *A. vexans.* The initial sporogony was not synchronized, the oocysts were present in 14 %, 28 % and 25 % of *C. p. pipiens* (6 dpi), *C. p. pipiens* f. *molestus* (10 dpi) and *A. vexans* (9 dpi), respectively. Mature oocysts were not seen. Sporozoites were absent from salivary glands of all exposed mosquitoes examined until 23 dpi. Thus, the sporogonic development of this parasite is abortive in all investigated mosquitoes.

3.6.2. Discussion

Plasmodium homocircumflexum lineage COLL4 was recorded in naturally infected birds belonging to Laniidae, Muscicapidae, Thraupidae, Furnariidae, Icteridae, Mimidae, and Fringillidae families (Durrant et al., 2006; Pérez-Tris et al., 2007), but its vectors remain unknown.

Culex p. pipiens is the most common European vector of avian malaria (Santiago-Alarcon et al., 2012; Valkiūnas, 2005), however this study shows that it is not a vector of *P. homocircumflexum.* Another widespread mosquito species, i.e. *A. vexans* and *C. p. pipiens* f. *molestus* are also resistant to this parasite. The exflagellation, fertilization followed by the formation of ookinetes and oocysts take place in all investigated mosquitoes (Fig. 13), but the sporogonic development is abortive on the oocyst stage.

Valkiūnas et al. (2013c) showed that closely related haemosporidian parasites belonging to *Haemoproteus* are able to develop ookinetes and oocysts throughout entire body of mosquito *Ochlerotatus cantans*, but development aborts and melanisation of oocysts occurs approximately 11 dpi. Abortive parasites development can occur at any stage of development in vector. The abortive development of *Plasmodium* spp. is commonly observed both in dipteran and vertebrate-hosts (Billingsley and Rudin, 1992). These examples show that 1) positive PCR signals and 2) the presence of ookinetes and oocysts of haemosporidian parasites cannot be considered as the final prove for vector determination. Observation of sporozoites is essential for determination of susceptible vectors. Thus, some dipteran species, which previously considered to be susceptible vectors based solely on observation of ookinetes and oocysts, might be not true vectors and this information should be reconsidered (see review by Santiago-Alarcon et al., 2012). This needs further experimental investigations.

CONCLUSIONS

1. The patterns of sporogonic development of the genetically similar and closely related cytochrome *b* lineages of *Plasmodium relictum* (pSGS1 and pGRW11) and different isolate of the lineage pSGS1 are the same.

2. Sporogony of the lineages pSGS1 and pGRW11 of *P. relictum* completes and sporozoites develop in *Culex pipiens pipiens* form *molestus.* This widespread mosquito should be taken in consideration in studies of avian malaria epidemiology.

3. *Haemoproteus minutus* and *H. belopolskyi* complete sporogony and produce sporozoites in the biting midge *Culicoides impunctatus,* which is an important vector of avian haemoproteids and worth more attention in epidemiology research of avian haemoproteosis.

4. Initial sporogonic development of *Haemoproteus* parasites occurs throughout the entire body of infected bird - biting mosquitoes *Ochlerotatus cantans*, but the sporogony is abortive.

5. Heavy *Haemoproteus* infections are markedly virulent to blood - sucking dipteran insects and cause mortality of these insects.

6. DNA of *Haemoproteus* parasites is detectable in the head, thorax and abdomen of infected mosquitoes *Ochlerotatus cantans* for several weeks following an infected blood meal, indicating the relatively long - time persistence of these parasites in resistant insects. That makes obstacles in application of solely PCR - based detection methods in determining vectors of haemosporidian parasite, particularly in wildlife.

7. The sporogonic development of *Plasmodium homocircumflexum* (lineage pCOLL4) occurs to the oocyst stage in the mosquitoes *Culex pipiens pipiens, Culex pipiens pipiens* form *molestus* and *Aedes vexans,* but sporozoites do not develop, indicating the abortive parasite sporogony.

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LIST OF PUBLICATIONS ON THE DISSERTATION TOPIC

1. **Kazlauskienė, R**., Bernotienė, R., Palinauskas, V., Iezhova, T.A., Valkiūnas, G. 2013. *Plasmodium relictum* (lineages pSGS1 and pGRW11): complete synchronous sporogony in mosquitoes *Culex pipiens pipiens*. Experimental Parasitology 133, 454 – 461.

2. Palinauskas, V, **Žiegytė, R**., Ilgūnas, M., Iezhova, T.A., Bernotienė, R., Bolshakov, C., Valkiūnas, G. Description of first cryptic avian malaria parasite *Plasmodium homocircumflexum* n. sp., with 2 experimental data on its virulence and development in avian hosts and mosquitoes. International Journal for Parasitology, accepted to press. 10.1016/j.ijpara.2014.08.012.

3. Valkiūnas, G., **Kazlauskienė, R**., Bernotienė, R., Palinauskas, V., Iezhova, T.A. 2013. Abortive long - lasting sporogony of two *Haemoproteus* species (Haemosporida, Haemoproteidae) in the mosquito *Ochlerotatus cantans*, with perspectives on haemosporidian vector research. Parasitology Research, 112 2159 – 2169.

4. Valkiūnas, G., **Kazlauskienė, R**., Bernotienė, R., Bukauskaitė, D., Palinauskas, V., Iezhova, T.A. 2014. *Haemoproteus* infections (Haemosporida, Haemoproteidae) kill bird - biting mosquitoes. Parasitology Research 113, 1011 – 1018.

5. **Žiegytė, R**., Bernotienė, R., Bukauskaitė, D., Palinauskas, V., Iezhova, T., Valkiūnas, G. 2014a. Complete sporogony of *Plasmodium relictum* (lineages pSGS1 and pGRW11) in mosquito *Culex pipiens pipiens* form *molestus,* with implications to avian malaria epidemiology. Journal of Parasitology, accepted to PubMed PMID: 24979183.

6. **Žiegytė, R**., Palinauskas, V., Bernotienė, R., Iezhova, T.A, Valkiūnas, G. 2014b. *Haemoproteus minutus* and *Haemoproteus belopolskyi* (Haemoproteidae): Complete sporogony in the biting midge *Culicoides impunctatus* (Ceratopogonidae), with implications on epidemiology of haemoproteosis. Experimental Parasitology 145, 74 – 79.

ABSTRACTS OF PRESENTATIONS AT SCIENTIFIC CONFERENCES

1. Bernotienė, R., **Kazlauskienė, R**. 2011. Entomological studies of blood-sucking dipteran insects (Diptera) as a basis for future vector studies of haemosporidian parasites (Haemosporida) in the Baltic region. Conference *Disease vectors today: Changes in ecology, climate and public health risks.* Kipi - Koovi Hiking Centre, Saaremaa, Estonia. Abstract book, 7 p.

2. Kazlauskienė, R., Bernotienė, R., Palinauskas, V., Iezhova, T.A., Valkiūnas, G. 2013. Long - lasting survival of *Haemoproteus* parasites in resistant blood-sucking insects, with perspectives on haemosporidian vector research. International Conference on Malaria and Related Haemosporidian Parasites of Wildlife, Vilnius, Lithuania. Abstract book, 63 p.

3. Kazlauskienė, R., Bernotienė, R., Bukauskaitė, D., Palinauskas, V., Iezhova, T.A., Valkiūnas, G. 2013. Complete synchronous sporogony of the malaria parasite *Plasmodium relictum* (lineages pSGS1 and pGRW11) in mosquitoes of the *Culex pipiens* group. The 8th European Congress on Tropical Medicine and International Health & 5th Conference of the Scandinavian - Baltic Society for Parasitology, Copenhagen, Denmark. Abstract, 230 p.

Note: The author's surname Kazlauskienė was changed to Žiegytė in 2013.