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Exo-erythrocytic stages of *Haemoproteus* (Apicomplexa, Haemosporida) parasites in wild birds: insights into developmental patterns

DOCTORAL DISSERTATION

Natural Sciences,
Zoology N 014

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VILNIAUS UNIVERSITETAS
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Egzoerithrocitinės *Haemoproteus* (Apicomplexa, Haemosporida) parazitų vystymosi stadijos laukiniuose paukščiuse: įžvalgos į vystymosi dėsningumus

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CONTENT

ABBREVIATIONS

BCIP – 5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt
BLAST – basic local alignment search tool
Bp – base pair
CISH – chromogenic *in situ* hybridization
Cyt b – mitochondrial cytochrome *b* gene
DNA – deoxyribonucleic acid
H&E – haematoxylin and eosin
NBT – nitro blue tetrazolium chloride
PCR– polymerase chain reaction
RNA – ribonucleic acid
SSC – sodium salt chloride

GLOSSARY

Asexual reproduction: a new offspring is produced by a single parent; there is no fusion of gametes.

Bayesian inference of phylogeny: The algorithm MCMC (Markov Chain Monte Carlo) 'move' in different directions, visit many trees, test their likelihood with combining the previous probability to generate the posterior probability. The tree presented at the end is the one which was the most visited and is the most likely explained by the sequences.

Cryptic species: species that cannot be distinguished using morphological characters despite molecular evidence that they are specifically distinct.

Erythrocytic meront: stage of the parasite that multiply asexually in the erythrocytes of the host, for example in *Plasmodium* species. *Haemoproteus* parasites do not have erythrocytic meronts.

Exo-erythrocytic meront (referred as meront): small (usually less than 100 μm in diameter) exo-erythrocytic stage, which is covered by a thin wall.

Exo-erythrocytic stage: parasite's asexual stage of development in tissues in its vertebrate host.

Gametocyte: intracellular blood stage of the parasite in its vertebrate host, which possesses sexual potency and is infective for vectors.

Haemo18*S* probe: oligonucleotide *Haemoproteus* genus-specific probe targeting the *18S* ribosomal RNA of the parasite.

Host: an organism that serves as a habitat for a parasite to complete at least a part of its life cycle.

Intensity of parasitemia: proportion of infected blood cells to total blood cells, usually expressed in percentage.

Lineage: genetic identity of avian haemosporidian parasites based on a barcoding DNA sequence of 478 bp of the cytochrome *b* gene.

Megalomeront: big (usually over 100 μm in diameter) exo-erythrocytic stage, which is covered by a capsular-like wall. This study distinguished the following stages of megalomeronts development: i) very young (cytomeres not distinguishable, size usually less than 50 μm, the host cell nucleus is visible); ii) developing (size usually over 50 μm, cytomeres can be present or not, but the host cell nucleus is absent); iii) mature (size usually over 100 μm, cytomeres no longer present but the merozoites are visible); iv) ruptured (capsular-like wall is ruptured in one or several places, merozoites can be present or have completely been released and only the capsular-like wall remains).

Morphospecies (referred as species): in haemosporidian parasites, a species that was described and characterized based mainly on morphological and limited host-specificity characters.

Parasitemia: presence of the parasites in blood usually sampled from the peripheral circulation.

Sexual reproduction: fusion of the female and male gametes resulting in a zygote.

LIST OF PUBLICATIONS ON THE DISSERTATION TOPIC

The present dissertation is based and written on the following original articles published to journals with an impact factor and referred in the Clarivate Analytic Web of Science database. The publications are referred using the Roman numerals throughout the dissertation:

I. **Duc, M.**, Ilgūnas, M., Valkiūnas, G. 2020. Patterns of *Haemoproteus majoris* (Haemosporida, Haemoproteidae) megalomeront development. *Acta Tropica*. 212, 105706. doi: 10.1016/j.actatropica.2020.105706 (Q1 quartile; IF: 3.112).

II. **Duc, M.**, Ilgūnas, M., Kubiliūnaitė, M., Valkiūnas, G. 2021. First report of *Haemoproteus* (Haemosporida, Haemoproteidae) megalomeronts in the brain of an avian host, with description of megalomerogony of *Haemoproteus pastoris*, the blood parasite of the common starling. *Animals*. 11(10), 2824. doi: 10.3390/ani11102824. (Q1 quartile; IF: 3.231).

III. Hernández-Lara, C., **Duc, M.**, Ilgūnas, M., Valkiūnas, G. 2021. Massive infection of lungs with exo-erythrocytic meronts in European robin *Erithacus rubecula* during natural *Haemoproteus attenuatus* haemoproteosis. *Animals*. 11(11), 3273. doi: 10.3390/ani11113273. (Q1 quartile; IF: 3.231).

IV. **Duc, M.**, Himmel, T., Ilgūnas, M., Eigirdas, V., Weissenböck, H., Valkiūnas, G. 2023. Exo-erythrocytic development of two *Haemoproteus* species (Haemosporida, Haemoproteidae), with description of *Haemoproteus dumbbellus*, a new blood parasite of bunting birds (Emberizidae). *International Journal for Parasitology*. *In press*. doi: 10.1016/j.ijpara.2023.02.009. (Q1 quartile; IF: 4.0).

V. **Duc, M.,** Himmel, T., Harl, J., Iezhova, T., Nedorost, N., Matt, J., Ilgūnas, M., Weissenböck, H., Valkiūnas, G. 2023. Comparative analysis of the exo-erythrocytic development of five lineages of *Haemoproteus majoris*, a common haemosporidian parasite of European passeriform birds. *Pathogens*. 12(7), 898. doi: 10.3390/pathogens12070898. (Q2 quartile; IF: 3.7).

AUTHOR CONTRIBUTIONS IN THE CORRESPONDING PAPERS

All authors have read and approved of the published version of the manuscripts.

I. Conceptualization: VG (Valkiūnas Gediminas); Methodology: IM (Ilgūnas Mikas), VG; Data curation: **DM** (**Duc Mélanie**); Formal analysis: **DM**; Investigation: **DM**, IM; Validation: IM, VG; Funding acquisition: VG; Supervision: VG; Writing – original draft: **DM**; Writing – review and editing: IM, VG.

II. Conceptualization: **DM**, VG; Data curation: **DM**; Investigation: **DM**, KM (Kubiliūnaitė Monika); Methodology: **DM**, IM, VG; Resources: VG; Visualization: **DM**; Funding acquisition: VG; Supervision: VG; Writing – original draft: **DM**, VG; Writing – review and editing: **DM**, IM, KM, VG.

III. Conceptualization: VG; Sample collection and preparation: **DM**, IM, VG; Phylogenetic analysis: H-LC (Hernández-Lara, Carolina); Microscopic examination: H-LC; Funding acquisition: VG, H-LC; Writing – original draft preparation: H-LC, VG, IM, **DM**; Writing – review and editing: H-LC, IM, **DM**, VG.

IV. Study conception and design: **DM**, HT (Himmel Tanja), WH (Weissenböck Herbert), VG; Sample collection: **DM**, IM, EV (Eigirdas Vytautas), VG, WH; Molecular analysis: **DM**, HT; Histology, chromogenic *in situ* hybridization and microscopy: **DM**, HT; Blood film microscopy examination: **DM**, VG; Writing – original draft: **DM**, HT; Writing – review and editing: **DM**, HT, IM, EV, WH, VG; Funding acquisition: WH, VG.

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LIST OF CONFERENCE PRESENTATIONS ON THE SUBJECT OF THE DISSERTATION

1. **Duc M.**, Ilgūnas M., Valkiūnas G. September 14-15, 2020. Patterns of *Haemoproteus majoris* (Haemosporida, Haemoproteidae) megalomeronts development. *International Online Conference on Blood Parasites of Wildlife*. Oral presentation.
2. **Duc M.**, Ilgūnas M., Valkiūnas G. April 21-23, 2021. Formerly neglected avian haemoproteosis: megalomeronts of *Haemoproteus majoris* develop in different bird species over different seasons. *9th Conference of the Scandinavian – Baltic Society for Parasitology (SBSP)*. Oral presentation.
3. **Duc M.**, Himmel T., Weissenböck H., Valkiūnas G. August 21-26, 2022. New data on exo-erythrocytic development of neglected avian *Haemoproteus* blood parasites (Haemoproteidae, Apicomplexa). *15th International Congress of Parasitology (ICOPA)*. Copenhagen, Denmark. Oral presentation.
4. **Duc M.**, Ilgūnas M., Weissenböck H., Valkiūnas G. September 5-8, 2022. Meronts and megalomeronts in avian *Parahaemoproteus* species, which is which and where do they develop? *5th International Conference on Malaria and Related Haemosporidian Parasites of Wildlife*. Bielefeld, Germany. Poster presentation.
5. **Duc M.**, Himmel T., Hernández-Lara C., Ilgūnas M., Weissenböck H., Valkiūnas G. September 11-15, 2022. Data on neglected avian haemoproteosis: exo-erythrocytic development of *Haemoproteus* species in naturally infected birds. *4th International Congress on Parasites of Wildlife (ICPOW)*. Kruger National Park, South Africa. Oral presentation.
6. **Duc M.**, Valkiūnas G. December 1-2, 2022. Exo-erythrocytic stages of avian *Parahaemoproteus* (Haemosporida, Apicomplexa) protists: how we study their diversity. *Protistology-UK autumn meeting*. Natural History Museum, London, United Kingdom. Oral presentation.
7. **Duc M.**, Himmel T., Ilgūnas M., Vytautas E., Weissenböck H., Valkiūnas G. April 24-27, 2023. Chromogenic *in situ* hybridization in avian haemosporidian research: how it further strengthens the research on tissue stages. *The Coins 2023*. Vilnius, Lithuania. Oral presentation.
8. **Duc M.**, Himmel T., Ilgūnas M., Weissenböck H., Valkiūnas G. June 5-7, 2023. *Haemoproteus majoris* exo-erythrocytic stages across its different lineages and avian hosts. *10th Conference of the Scandinavian-Baltic Society for Parasitology (SBSP)*. Tartu, Estonia. Oral presentation.

AWARDS

1. Scandinavian – Baltic Society for Parasitology (SBSP) Student Participation Grant to attend the *9th Conference of the Scandinavian – Baltic Society for Parasitology.* Online conference, April 21-23, 2021.
2. Travel grant to attend the *5th International Conference on Malaria and Related Haemosporidian Parasites of Wildlife.* The organizers provided support to attend the conference in Bielefeld, Germany, September 5-8, 2022.
3. Research Council of Lithuania. Financial support to attend the *4th International Congress on Parasites of Wildlife (ICPOW)* in the Kruger National Park, South Africa, September 11-15, 2022. (no. S-DAK-22-40).
4. International Society of Protistology (ISOP) financial support to attend the conference *Protistology-UK autumn meeting* in the Natural History Museum, London, United Kingdom, December 1-2, 2022.
5. EU SYNTHESYS+ grant to work at the Natural History Museum (NHM, London, United Kingdom) to access the haemosporidians collection (Protists) with the application titled "Comparative research on exo-erythrocytic development of wildlife haemosporidian parasites" (GB-TAF-TA4-005).
6. EU SYNTHESYS+ grant to work at the Musée National d'Histoire Naturelle (MNHN, Paris, France) to access the haemosporidians collection (Protists) with the application titled "Comparative research on exo-erythrocytic development of wildlife haemosporidian parasites" (FR-TAF_Call4_062).
7. Research Council of Lithuania. Scholarship for academic achievements (2022, no. P-DAP-23-164).
8. "1st place for the best Oral presentation at The Coins 2023 conference", Vilnius, Lithuania.

Haemosporidian parasites (Apicomplexa, Haemosporida) are endoparasites of reptiles, birds, and mammals (Garnham, 1966; Valkiūnas, 2005; Telford Jr., 2009). Haemosporidians of birds are found all over the globe, except in Antarctica (Clark et al., 2014). They are classified in four families – the Plasmodiidae, Garniidae, Haemoproteidae, and Leucocytozoidae (Valkiūnas, 2005) – and their genetic lineages are recorded in over 2195 species of birds (MalAvi database (Bensch et al., 2009)). These obligate heteroxenous pathogens occur in two hosts: an avian host where the asexual multiplication occurs and the gametocytes (invasive for the vector) develop, and a dipteran vector where the sexual reproduction occurs and sporozoites develop (invasive stage for the avian host) (Valkiūnas, 2005; Valkiūnas and Atkinson, 2020). Two subgenera of the genus *Haemoproteus* Kruse, 1890 are recognised – *Haemoproteus* Kruse, 1890 and *Parahaemoproteus* Bennett, Garnham and Fallis, 1965. Louse flies (Diptera: Hippoboscidae) transmit parasites of *Haemoproteus*, whereas biting midges of *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) transmit parasites of *Parahaemoproteus* (Valkiūnas, 2005; Bukauskaitė et al., 2019b; Valkiūnas and Atkinson, 2020). The latter subgenus represents the majority of described haemosporidian species and genetic lineages as well as being the focus for this study.

Haemoproteus parasites and other haemosporidians infect their avian host only through the sporozoite stage during active bites of infected vectors and enter the peripheral circulation. The parasite penetrates various host cells, transforming into unicellular trophozoites and then into exo-erythrocytic stages (meronts and/or megalomeronts). After maturation of the latter, the host cell rupture, and the merozoites are released, invading the erythrocytes to develop into gametocytes or penetrating other tissue cells to continue the asexual multiplication in organs. The gametocytes produce gametes in the vectors (Valkiūnas, 2005; Valkiūnas and Atkinson, 2020).

The description of new species of *Haemoproteus* is currently based on the morphological characters of the gametocytes and their influence on the host cells from blood films microscopy (Valkiūnas, 2005; Valkiūnas and Iezhova, 2022). The limited experimental data about vertebrate-host specificity can also be used (Bukauskaitė et al., 2019b; Chagas et al., 2019). The genetic assessment of the parasite is commonly based on the partial mitochondrial cytochrome *b* (*cyt b*) gene sequence, defined by 478 bp as a barcoding region for the parasites ((Bensch et al., 2000; Hellgren et al., 2004), MalAvi database (Bensch et al., 2009)) and is referred as a lineage. A new

lineage is defined when at least one base pair differs in the barcoding region from any other known sequence (Bensch et al., 2009).

Formerly considered as relatively harmless to their avian host in comparison to closely related *Plasmodium* Marchiafava and Celli, 1885 and *Leucocytozoon* Berestneff, 1904 parasites (Bennett et al., 1993), parasites of the genus *Haemoproteus* have frequently been neglected in parasitology and veterinary medicine, and their life cycle remains unknown for a great majority of described species and lineages. To date, 178 species of *Haemoproteus* have been described (Valkiūnas and Iezhova, 2022; Duc et al., 2023), while 1904 lineages have been identified. However, only 161 lineages have their species described, and only 78 described species are characterized molecularly (MalAvi database (Bensch et al., 2009)).

Numerous parasite descriptions date before the establishment of PCR protocols in avian haemosporidian research, lacking the genetic molecular characterization (Aragão, 1908; Farmer, 1964; Baker, 1966; Khan and Fallis, 1969; Peirce, 1976; Ahmedt and Mohammed, 1977; Miltgen et al., 1981; Atkinson et al., 1986; Atkinson and Forrester, 1987; Atkinson et al., 1988; Earle et al., 1993; Cardona et al., 2002; Paperna and Gill, 2003; Peirce et al., 2004). Molecular studies often do not include investigation of blood films, as pointed out by Clark et al. (2014) and Bernotienė et al. (2016). This rapidly increased the gap between species taxonomic diversity and the molecular diversity of these parasites. Before 2019, exo-erythrocytic stages (meronts and/or megalomeronts) were known for 15 species of *Haemoproteus* (Valkiūnas and Iezhova, 2017), mainly from old publications. The true diversity and specificity of these parasites on tissue stages remains unknown, and phylogenetic analyses could not explain features of their exo-erythrocytic stage development due to the lack of information on most described species.

Recently, Ortiz-Catedral et al. (2019) and Himmel et al. (2019) reported infections of *Haemoproteus* (*Parahaemoproteus*) in dead parrots, with numerous megalomeronts found in clusters in the heart and other organs. The stages were of similar morphology in these two studies, and the *Haemoproteus* identity of the tissue stages were proved by the development and application of the genus-specific molecular diagnostic chromogenic *in situ* hybridization (CISH) (Himmel *et al.*, 2019). Ortiz-Catedral et al. (2019) reported the mortality of parrots belonging to 12 species that do not usually get these infections in wildlife; the parasites were molecularly identified as *Haemoproteus minutus* Valkiūnas and Iezhova, 1992 and *Haemoproteus asymmetricus* Valkiūnas, Ilgūnas, Bukauskaitė, Duc and Iezhova, 2021, common parasites of thrushes (Passeriformes: Turdidae). These findings raised the questions if the haemoproteosis and tissue stages characteristics

reported in unusual avian hosts are due to the parasite development in non-adapted hosts and represent possible abortive infections, or if the found megalomeronts are the parasite-species features. It is still unclear if the cycle of exo-erythrocytic development is a feature of a parasite species or a hosts-species or both. The following health questions should also be considered in regard to avian haemoproteosis: i) how harmful these parasites are to birds, ii) how and in which organs the parasites preferably develop, and iii) do the generalist and specialist parasites develop similarly?

The investigation of tissue stages requires sampling the host internal organs from naturally dead individuals or after euthanasia. Parasitemia is a good indicator of infection in birds (Valkiūnas, 2005; Valkiūnas and Atkinson, 2020). It can be determined (Valkiūnas and Iezhova, 2022) and the intensity of parasitemia can be calculated (Godfrey et al., 1987) during microscopic examinations of blood films. However, it remains unclear how the intensity of parasitemia is related to the intensity and patterns of the exo-erythrocytic development in the host. A low parasitemia could indicate two different events: i) the exo-erythrocytic stages are still developing, and only a few have matured and released merozoites, which have invaded the erythrocytes and developed into gametocytes; or ii) the exo-erythrocytic stages have ruptured before sampling, and the tissue stages remaining in the organs would be few, resulting in difficulties to observe them. Similarly, a high parasitemia could be because i) the majority of exo-erythrocytic stages were matured and ruptured but could still be present and observed in the organs, or ii) all tissues stages have ruptured and released the merozoites recently and would be difficult to find in the organs. Targeted research and experiments on relationship between parasitemia and exo-erythrocytic development intensities are needed to resolve those questions.

Most published reports about tissue stages of haemoproteids are case observations, which provide information only about a single point of the parasite development – at the host death – corresponding to a stop in the development of the parasite. There are only two experimental studies (Atkinson et al., 1986; Atkinson et al., 1988) in which the exo-erythrocytic development of *Haemoproteus* parasites was targeted during the entire course of infection, providing data about a pattern of development within one host species infected with one parasite species through time. Other experiments on this subject are absent, revealing the difficulty of experimental research of *Haemoproteus* species exo-erythrocytic development, as sporozoite-induced infections are needed and are challenging, particularly in regard to the parasites of wild birds (Bukauskaitė et al., 2019a). The transfusion of infected bird blood to another uninfected host is non-effective and cannot be used in

Haemoproteus species experimental research, a clear difference with avian parasites of *Plasmodium* experimental research (Garnham, 1966). This increases the value of field observations in this branch of parasitology, particularly to study exo-erythrocytic stages of *Haemoproteus* species, which are accessible by sampling naturally infected wild birds. This opportunity was explored in this study by investigating birds of the order Passeriformes.

OBJECTIVE AND MAIN TASKS OF THE STUDY

The objective of this study was to expand the knowledge about the exo-erythrocytic development of avian haemosporidian parasites belonging to the genus *Haemoproteus* (subgenus *Parahaemoproteus*) (Haemosporida, Haemoproteidae) using naturally infected wild birds.

To achieve this objective, the following tasks were set:

1. To investigate and conduct comparative analysis of exo-erythrocytic stages morphology and site of development of the parasites *Haemoproteus majoris*, *Haemoproteus pastoris*, *Haemoproteus hirundinis*, *Haemoproteus attenuatus*, and *Haemoproteus dumbbellus* in their corresponding natural avian host.
2. To determine the use of the intensity of parasitemia in choosing the host individuals to investigate exo-erythrocytic stages of *Haemoproteus* parasites in naturally infected birds.
3. To apply the chromogenic *in situ* hybridization method to confirm the genus identity and the presence of the exo-erythrocytic stages of *Haemoproteus* parasites.
4. To determine if the data on morphospecies and the phylogenetic analyses based on partial *cyt b* gene can predict *Haemoproteus* parasites exo-erythrocytic stages morphology and/or localization in organs for patterns of development in naturally infected birds.

STATEMENTS TO BE DEFENDED

1. *Haemoproteus majoris*, *Haemoproteus pastoris* and *Haemoproteus hirundinis* develop megalomeronts of different morphologies and in different organs.
2. *Haemoproteus attenuatus* and *H. dumbbellus* develop meronts mainly in the lungs; but no megalomeronts in the investigated individuals.
3. Exo-erythrocytic stages of *Haemoproteus* parasites also develop in the brain, with first reports of cerebral haemoproteosis in *H. pastoris, H. majoris* and *H. dumbbellus*.
4. The intensity of parasitemia is not associated with the exo-erythrocytic stages presence in the organs during *H. majoris*, *H. pastoris*, *H. hirundinis*, *H. attenuatus* and *H. dumbbellus* natural infections.
5. The chromogenic *in situ* hybridization method confirms the generic identity of the found exo-erythrocytic stages of *Haemoproteus* parasites and helps to find the small tissue stages in the organs.
6. The currently available data on the exo-erythrocytic stages coupled with phylogenetic analysis based on the partial *cyt b* gene sequences are insufficient to predict the development of avian parasites of the genus *Haemoproteus* in tissues of the host. However, closely related lineages of parasite morphospecies are predictable in this regard.
7. All studied morphospecies of *Haemoproteus* have morphologically distinct tissue stages, indicating its value for parasite species taxonomy.

NOVELTY AND RELEVANCE OF THE RESEARCH

1. Exo-erythrocytic development showed parasite species-specificity. *Haemoproteus majoris*, *H. pastoris* and *H. hirundinis* developed only megalomeronts, while *H. attenuatus* and *H. dumbbellus* developed only meronts in the investigated individuals.
2. All examined species of *Haemoproteus* showed markedly different site of development (organ) for tissue stages, indicating the need of studying as many organs as possible in research of exo-erythrocytic development in still non-studied parasite species.
3. Cerebral haemoproteosis was reported for the first time, with meronts and megalomeronts of *Haemoproteus* species found to develop in the brain, indicating a formerly unknown pathway of pathology.
4. Very young megalomeronts were found for the first time, and for different lineages of *H. majoris* in spring, summer, and autumn. It remains unknown if these stages persist during winter.
5. The selection of individuals for exo-erythrocytic stages investigations should not be solely based on the intensity of parasitemia of the parasite infection.
6. Haemosporidian exo-erythrocytic research should investigate both haematoxylin and eosin-stained sections (for structure, morphology) and chromogenic *in situ* hybridization treated sections (for small, irregularly shaped stages and to confirm the generic identity of the stages).
7. Three lineages were assigned to their morphospecies for the first time: hEMCIR01 to the new species *H. dumbbellus*, hDELURB2 to *H. hirundinis,* and hCWT4 to *H. majoris*. The molecular characterization of these parasites is important for broadening *Haemoproteus* species molecular research opportunities and further study potential phylogenetic patterns of development among haemoproteid parasite species.
8. The available data remain scarce to answer the question if phylogenetic analysis can predict the development of exo-erythrocytic stages in distinct parasite *Haemoproteus* species on a larger scale.
9. Parasites of closely related lineages of *H. majoris* developed similarly in their respective avian hosts, indicating that lineage information holds preliminary prediction of possible morphology and localization of tissue stages in still non-studied lineages of same morphospecies in different avian hosts.
10. Morphological features of exo-erythrocytic stages in studied parasites were consistent with morphospecies and are worth more attention in taxonomy of *Haemoproteus* parasite on species levels.

1. BRIEF LITERATURE REVIEW

Research on life cycle of haemosporidian parasites is essential to better understand the effects these pathogens can have on their host. However, different stages of the life cycle (exo-erythrocytic stages, gametocytes, gametes, ookinetes, oocysts, and sporozoites) of *Haemoproteus* parasites remain unknown for most described species (Valkiūnas, 2005; Valkiūnas and Iezhova, 2017; Bukauskaitė et al., 2019b). This is particularly true for exo-erythrocytic development (Valkiūnas and Iezhova, 2017). Tissue stages of *Haemoproteus* parasites can be distinguished from the more studied and closely related *Plasmodium* parasites by the development of large-size exo-erythrocytic stages in the organs of the hosts, the megalomeronts (> 100 μm in diameter) (Valkiūnas and Iezhova, 2017; Groff et al., 2019; Himmel et al., 2019, 2021; Ilgūnas et al., 2019, 2022; Ortiz-Catedral et al., 2019), or the agglomerations of meront (around 50-70 μm) (Hernández-Lara et al., 2021). Previous studies (Aragão, 1908; Anschüzt, 1909; Farmer, 1964; Baker, 1966; Khan and Fallis, 1969; Peirce, 1976; Ahmedt and Mohammed, 1977; Miltgen et al., 1981; Sibley and Werner, 1984; Atkinson et al., 1986; Atkinson and Forrester, 1987; Atkinson et al., 1988; Earle et al., 1993; Cardona et al., 2002; Garvin et al., 2003; Paperna and Gill, 2003; Peirce et al., 2004) could only rely on blood films microscopy for the determination and confirmation of the parasite species identity, while nowadays molecular methods can confirm the presence of the parasite and its genetic diversity (Himmel et al., 2019, 2021; Ilgūnas et al., 2019, 2022).

Scarcely studied during the 20th century, *Haemoproteus* parasites have gained particular attention after several reports of mortalities in non-adapted hosts (parrots) (Olias et al., 2011; Himmel et al., 2019; Ortiz-Catedral et al., 2019), with reports of numerous exo-erythrocytic stages being responsible for marked pathology in the heart, lungs, muscles, and gizzard. Such reports were available in previous literature (Aragão, 1908; Atkinson and Forrester, 1987; Earle et al., 1993; Paperna and Gill, 2003), but there were no proofs that the tissue stages certainly belonged to *Haemoproteus* parasites. It still remains unknown how these and closely related parasites develop in their natural avian host. However, few reports from wild birds are available that confirm the infection with several approaches (Groff et al., 2019; Himmel et al., 2019, 2021; Ilgūnas et al., 2019, 2022). The presence of gametocytes (blood infection, parasite identification) and exo-erythrocytic stages (organs infection) were investigated in parallel with the molecular characterization of the parasite (lineage identification).

In 2019, Himmel et al. (2019) used the chromogenic *in situ* hybridization (CISH) technique with designed *18S* oligonucleotide probes to target specifically and independently parasites ribosomal-RNA of three haemosporidian genera. It ensures the generic identification of tissue stages, particularly during possible co-infections, which are common in wildlife (*Haemoproteus* with *Leucocytozoon* or *Plasmodium*) (Himmel et al., 2021; Ilgūnas et al., 2022). If RNA is expressed, CISH signals should be visible, providing opportunities to distinguish between the parasites belonging to different genera (Dinhopl et al., 2011; Himmel et al., 2019). The technique confirms the genus of the visible parasite – gametocytes in the blood streams of the organs, exo-erythrocytic stages in the organs – and simplifies the identification of unusually shaped parasites morphology (which are difficult to assume belonging to *Haemoproteus* parasites) and/or small stages, which can be difficult to recognize in histological preparations. This technique was used on all studied species.

Records of exo-erythrocytic stages of different species of *Haemoproteus*, for which molecular results were obtained, have increased since the publication by Himmel et al. (2019), contributing to the knowledge on the development of *Haemoproteus* parasites in tissues (Himmel et al., 2019, 2021; Ilgūnas et al., 2019, 2022). Material of these studies were either from bird individuals found naturally dead (Himmel et al., 2019, 2021; Ilgūnas et al., 2022) or from purposely targeted individuals infected with a specific parasite species (Ilgūnas et al., 2019). During investigation of wildlife birds, the parasitemia is estimated by the observation of gametocytes in blood films (Valkiūnas, 2005), but it has not been addressed if the intensity of parasitemia might be considered as a good indicator for exo-erythrocytic stages presence in the organs. Indeed, investigating wild birds implicates not knowing when in the life cycle of the parasite the bird is accessed for research, as it could be at the beginning or at the end of the infection. This calls for sampling of individuals with different intensities of parasitemia in relation to the presence of exo-erythrocytic stages. This study addressed this issue using the species *Haemoproteus majoris* (Laveran, 1902), *Haemoproteus pastoris* Mello, 1935, *Haemoproteus hirundinis* (Sergent and Sergent, 1905), *Haemoproteus attenuatus* Valkiūnas, 1989 and *Haemoproteus dumbbellus* Duc, Himmel, Ilgūnas, Eigirdas, Weissenböck, Valkiūnas, 2023, in their natural hosts as research objects.

Similarly, it is still unknown if the development of exo-erythrocytic stages is more host species-specific or parasite species-specific. If exo-erythrocytic stages were to be host specific, differences in the parasite development would be expected (morphology and/or site of development and

intensity) for the same parasite species in different host species. However, if the exo-erythrocytic stages were to be parasite species-specific, no major differences would be expected to occur between exo-erythrocytic stages of the same parasite species in different host species, but differences would be expected in different parasites in different or same host species. These issues remain unclear and need targeted research. The available data currently point out to species-specific exo-erythrocytic development as all reported parasite species, in different host species, have different morphologies and in association of different organs (Groff et al., 2019; Himmel et al., 2019, 2021; Ilgūnas et al., 2019, 2022; Ortiz-Catedral et al., 2019). The study of one parasite species in different hosts would provide further insights into the problem, even more so if the species has several lineages recognized. This study addressed this issue using *H. majoris* and five of its lineages in natural hosts.

Phylogenetic trees can be used in vector research to help determine the development of the parasite in the vector (fast developing or slow developing (Chagas et al., 2019)) and the genus of the vector that transmits parasites of the genus *Haemoproteus* (Bukauskaitė et al., 2019b). Parasites are also known to be specialists or generalists to their avian hosts (Križanauskienė et al., 2006; Nilsson et al., 2016; Clark and Clegg, 2017; Ellis et al., 2020). However, it has not been addressed whether *Haemoproteus* parasites do develop both exo-erythrocytic stages (meronts and megalomeronts) or if each parasite species develops only one, nor if the specificity of a parasite could also point out to a different exo-erythrocytic development in their avian host. Exo-erythrocytic development might be species-specific, but actual patterns of development are difficult to draw with the current knowledge. Indeed, exo-erythrocytic stages are known for nine species (Anschüzt, 1909; Khan and Fallis, 1969; Miltgen et al., 1981; Sibley and Werner, 1984; Atkinson et al., 1986; Atkinson and Forrester, 1987; Atkinson et al., 1988; Cardona et al., 2002; Garvin et al., 2003; Peirce et al., 2004) which have not been molecularly characterized yet. It is therefore impossible to know their relationships with other parasite species for which genetic lineages are known. In other words, the available information is insufficient to understand if phylogenetic hypotheses can help to predict patterns and relationships between species and lineages within a species. More intensive studies on exo-erythrocytic stages of different species of *Haemoproteus* associated with their molecular characterization are necessary, as well as the description and recognition of species from blood films in complement to lineage descriptions. This study addressed this issue partially using five species of *Haemoproteus* as research objects.

Reviewing the different cases of exo-erythrocytic development of *Haemoproteus* parasites showed that only morphological or only molecular studies cannot address alone the questions to understand these parasites biology and taxonomy. Morphological research supplemented with molecular characterizations are necessary to better understand these parasites.

2. MATERIAL AND METHODS

Different methods were applied in this study. The research methodologies included the combination of fieldwork, microscopic examination and image analysis, DNA extractions, PCR amplifications, DNA sequencing and phylogenetic analysis, histology and chromogenic *in situ* hybridization, and statistical analysis. Detailed descriptions of the methods and research approaches are given in the published original papers (**Papers I-V**). Brief descriptions of main used research protocols are provided below.

2.1. Study sites

Samples were collected from 2016 to 2022 at two sites, using mist nests in the Labanoras Regional Park (55°12'25.77" N, 25°56'26.47" E) (**Paper I**) and stationary 'Rybachy type', zigzag traps and mist nets in the Ventės Ragas Ornithological station (55°20'38.93" N, 21°11'34.05" E) (**Papers II, III, IV, V**). The microscopic and molecular analyses as well as histological procedures were performed at the P.B. Šivickis Laboratory of Parasitology, Nature Research Centre, Vilnius, Lithuania (https://www.gamtostyrimai.lt) (**Papers I-V**) and at the Institute of Pathology, Department for Pathobiology, University of Veterinary Medicine Vienna, Vienna, Austria (https://www.vetmeduni.ac.at) (**Papers IV, V**). Representative blood films with gametocytes and preparations of histology sections with exo-erythrocytic stages were deposited at the Nature Research Centre (**Papers I-V**) and at the Queensland Museum, Brisbane, Australia (**Papers IV, V**), and are available on request.

2.2. Collection of samples

2.2.1. Blood sampling

Blood samples were collected by puncturing the brachial veins of birds and using a heparinized capillary. A drop of blood was used to prepare several blood films, which were air dried and fixed in methanol. One blood film was stained with 30% Giemsa for 15 min on site of the collection, and the microscopic examination was done straight away to identify the parasite species based on the gametocyte stages and to pre-estimate the intensity of parasitemia (**Papers I-V**) (Valkiūnas, 2005). The remaining blood was transferred into tubes with SET-buffer (0.05M Tris, 0.15M NaCl, 0.5M EDTA, pH=8.0) (Hellgren et al., 2004), stored at +4°C on site and at -20°C in

the laboratory. Molecular analyses were later conducted on these samples (cf. 2.5) (**Papers I-V**).

2.2.2. Organ sampling and processing

Based on the on-site microscopic examination of the blood films, a total of 52 single-infected and 11 co-infected bird individuals with infections of *Haemoproteus* were selected, euthanized and their organs retrieved (**Papers I-V**). Tissue samples from four dead birds were also used, two from the archive of the Institute of Pathology at the University of Veterinary Medicine Vienna (**Paper IV**) and two brough dead to the Nature Research Centre (**Paper V**). The following organs were collected: brain, heart, kidneys, liver, spleen, trachea, lungs, oesophagus, gizzard, intestine, pancreas, reproductive organs, and pectoral and leg muscles (**Papers I-V**). The organs were fixed in 10% formalin, rinsed in distilled water, dehydrated in a series of increased alcohol concentrations, cleared in xylene solvent, and embedded in paraffin wax.

2.3. Microscopy of blood films

The additional collected blood films were stained using a 10% Giemsa staining for 1h. These preparations were used for deposition in collections. Microscopic examination was done using a light microscope BX41TF (**Papers I, IV**), BX61 (**Paper II, V**), BX51 (**Paper III**) (Olympus, Tokyo, Japan) and the intensity of parasitemia was calculated by counting the number of parasites per 1000 erythrocytes or per 10000 erythrocytes in case of low parasitemia (Godfrey et al., 1987). Images and measurements were taken using an Olympus DP12 digital camera with the Olympus DP-SOFT software (**Papers I, II, IV**) or an Olympus DP70 digital camera with the AnalySIS FIVE software (**Paper V**) (Olympus, Tokyo, Japan); the ImageJ 1.53a software (National Institutes of Health, Bethesda, MD, USA, https://imagej.nih.gov/ij/USA) was used for parasite measurements (**Paper III**). Figures were prepared using the software CorelDraw 2019 (RRID:SCR_014235, https://www.coreldraw.com/en/) (**Papers I, II, IV**) and Adobe InDesign CS6 (Adobe, https://www.adobe.com/products/indesign.html) (**Paper V**).

Parasites species were identified and described (**Papers I-V**) based on standard morphological characters of the gametocytes and their host cells (Valkiūnas, 2005; Valkiūnas and Iezhova, 2022). Arithmetic means and standard deviation were calculated for the species description (**Paper IV**).

Morphological identifications were supported by DNA sequence information (cf. 2.5).

2.4. Histological techniques

2.4.1. Haematoxylin and eosin staining

Sections of 2 to 4 µm were cut from the paraffin blocks for histological analyses, using a Leica RM2245 microtome (Leica Biosystems, Inc. Buffalo Grove, USA). The sections were air dried or dried in an incubator for 30 min at 60°C, stained using H&E and covered with coverslips (**Papers I-V**).

Microscopy of exo-erythrocytic stages was done using a microscope BX41TF (**Papers I, II, IV, V**), or BX51 (**Papers III, IV, V**) at magnifications ×100 to ×1000 and images and measurements were taken using the cameras Olympus DP12, DP71 or UC90 (Olympus, Tokyo, Japan) with the software Olympus DP-SOFT or cellSens Entry (**Papers I-V**). The ImageJ 1.53a software was also used for measurements (**Papers III**, **IV**). Figures were prepared using the software CorelDraw 2019 (**Papers I, II, IV, V**).

2.4.2. Chromogenic *in situ* hybridization

Chromogenic *in situ* hybridization with the Haemo18*S* probe was additionally performed on sections cut at 2-3 µm (Himmel et al., 2019) (**Papers IV, V, unpublished data for parasites species of Papers II, III**) to confirm and expand the detection of the stages found in the corresponding organs in H&E-stained preparations. Briefly, the sections were dried in an incubator for 30 min at 60°C, deparaffinized in xylene, hydrated in a decreasing concentration of alcohol, went through proteolysis (opens the cell membrane and nucleus), incubated overnight with a hybridization solution (with the probe), washed for unspecific binding of the probe in SSC solutions. The sections were then immunologically proofed for hybridization with an equilibration buffer and an anti-Digoxigenin-AP-fab-fragment (antibody) solution, washed in different buffers, coloured for substrate with NBT and BCIP (to develop the signal), stained shortly with haematoxylin, and covered with a coverslip (Dinhopl et al., 2011; Himmel et al., 2019). Microscopic examinations were conducted as explained in 2.4.1.

2.5. Molecular analyses

2.5.1. DNA extraction, PCR, and sequencing

DNA was extracted from the blood samples stored in SET-buffer using an ammonium acetate protocol (Richardson et al., 2001) (**Papers I-V**). DNA was extracted from the frozen tissue samples of the Institute of Pathology archives and from blood samples using the DNeasy Blood & Tissue Kit (QIAGEN, Venlo, Netherlands) (**Papers IV, V**). Nested PCRs were performed to determine the specific lineages present in the infection. The outer primers pair HAEMNFI/HAEMNR3 and inner primer pair HAEMF/HAEMR2 (Bensch et al., 2000; Hellgren et al., 2004) were used following the original protocols to amplify DNA of *Plasmodium* and *Haemoproteus* parasites, while the inner primer pair HAEMFL/HAEMR2L (Hellgren et al., 2004) was used to amplify DNA of the *Leucocytozoon* parasites (**Papers I-V**). Samples failed to be amplified by these primers were screened by a second nested PCR using the primers pairs PLAS1F/HAEMNR3 and 3760F/HAEMJR4 (Beadell et al., 2004; Hellgren et al., 2004; Duval et al., 2007; Pérez-Rodríguez et al., 2013), which amplify DNA from representatives of the three genera (**Papers IV**).

A total of 2 µl of the final PCR products were used to check for positive amplifications on 2% agarose gels. The positive PCR products were prepared for sequencing and sequenced from both 5' and 3' ends with Big Dye Terminator V3.1 Cycle Sequencing Kit and ABI PRISM™ 3100 capillary sequencing robot (Applied Biosystems, Foster City, California) (**Papers I-V**) or were sent to the commercial sequencing company Microsynth (Microsynth, Austria) (**Paper IV, V**).

The chromatograms were checked in the software Geneious Prime 2020.0.5 or 2022.0.2 (Dotmatics, Auckland, New Zealand, https://www.geneious.com) (**Papers I, II, IV, V**), or in SnapGene Viewer 5.2.4 (Insightful Science, San Diego, CA, USA, www.snapgene.com) (**Paper III**), or in Bioedit (https://bioedit.software.informer.com) (Hall, 1999) (**Paper IV, V**). Single infections were confirmed by single peaks in the sequence chromatograms, whereas co-infections were characterized by multiple peaks. The obtained sequences were BLASTed in MalAvi database (Bensch et al., 2009) and in NCBI GenBank (National Library of Medicine, Bethesda, Maryland, https://www.ncbi.nlm.nih.gov/genbank/). All obtained sequences were deposited in GenBank (**Papers I-V**).

Genetic pairwise distances were calculated for the lineages of *H. majoris* (**Paper V**) with the software MEGA-X: Molecular Evolutionary Genetics

Analysis across computing platforms (Kumar et al., 2018) using the Maximum Composite Likelihood model of substitution.

2.5.2. Phylogenetic analysis

The *cyt b* DNA sequences were used in phylogenetic analyses. Parasite species characterized molecularly were selected for Bayesian phylogenetic tree calculations. Specifically, species for which the lineage(s) and exo-erythrocytic stage(s) are known were used in the analysis. Different species of *Haemoproteus*, *Parahaemoproteus* and *Plasmodium* were used, with one lineage of *Leucocytozoon* as outgroup (**Papers I-IV**). The phylogenetic model was selected through jModeltest-2.1.10 (Guindon and Gascuel, 2003; Darriba et al., 2012) (**Papers I-IV**). The analyses were run using the software Geneious Prime 2020.0.5 (**Papers I, II**) or 2022.0.2 (**Paper IV**) with the MrBayes plugin v3.2.6 (Huelsenbeck and Ronquist, 2001) or in MrBayes 3.2.7 (University of Rochester, Rochester, NY, USA; Evolutionary Biology Centre, Uppsala, Sweden) (Ronquist et al., 2012) (**Paper III**). A total of 5 to 15 million generations were run with 25% discarded as burn-in. The trees were visualized in Geneious Prime 2020.0.5, or 2022.0.2 (**Papers I, II, IV**) or in the FigTree 1.4.4 software (University of Edinburgh, Edinburgh, Scotland) (Rambaut, 2006) (**Paper III**).

2.6. Ethical statement

All procedures with the birds were according to permits and complies with the laws of Lithuania and Austria. Licenced researchers performed the handling of the birds and collection of samples, all approved by the Lithuanian Environmental Protection Agency, Vilnius (permits numbers: 2016 05 05 Nr. 23; 2017 04 26 Nr. 23; 2017 06 05 Nr. 33; 2018 04 13 Nr. 24; 2019 04 19 Nr. 23; 2020 04 08 Nr. (26)-A4E-2892 (2020 04 07 Nr. 21); 2021 05 05 Nr. (26)-SR-96, and 2022 04 28 Nr. (26)-SR-152) (**Papers I-V**). No additional ethical approval was necessary for the samples collected at the Institute of Pathology, as they were collected during the routine diagnostic services at the Vetmeduni Vienna (**Paper IV**).

3. RESULTS AND DISCUSSION

All results were described and discussed in the original articles and constitute the core of this dissertation (**Papers I-V**). The main results corresponding to the dissertation tasks are summarized below.

3.1. Megalomeronts development in three species of *Haemoproteus*: *Haemoproteus majoris*, *H. pastoris* and *H. hirundinis*

In all, 43 individuals from 14 bird species have been investigated for exo-erythrocytic stages of *H. majoris* (**Papers I, V**). All individuals positive for exo-erythrocytic stages were found with only megalomeronts in the different *H. majoris* infections: lineages hCCF5, hCWT4, hPARUS1, hPHSIB1, hPHYBOR04 and hWW2 (**Papers, I, V**, (Ilgūnas et al., 2019)). Megalomeronts of *H. pastoris* were found in four out of five *Sturnus vulgaris* Linnaeus, 1758 (Passeriformes: Sturnidae) (**Paper II**). Megalomeronts of *H. hirundinis* were found to affect only the pectoral muscles of two out of six *Delichon urbicum* Linnaeus, 1758 (Passeriformes: Hirundinidae) (**Paper IV Figure 5**) and were elongated rather than round compared to other known megalomeronts of *Haemoproteus* parasites (**Papers I, II, IV, V**, (Groff et al., 2019; Himmel et al., 2019, 2021; Ilgūnas et al., 2019, 2022; Ortiz-Catedral et al., 2019)). The cytomeres present in the megalomeronts were different between parasite species, more interconnected in *H. majoris* (**Paper I, Figures 3, 4; Paper V, Figures 2, 3, Figures S1-S5**, (Ilgūnas et al., 2019)), forming condensed masses within the structure in *H. pastoris* (**Paper II, Figures 3, 4**), or developed a star shape, with the merozoites budding from them in *H. hirundinis* (**Paper IV, Figure 5**). In these three parasite species, the found megalomeronts were of similar morphology across the different bird individuals but differed between species of parasites of this study (**Papers I, II, IV, V**) and from the literature (Farmer, 1964; Miltgen et al., 1981; Atkinson et al., 1986; Groff et al., 2019; Himmel et al., 2019, 2021; Ilgūnas et al., 2019, 2022; Ortiz-Catedral et al., 2019).

Megalomeronts found in *H. majoris* hCCF5 lineage infections (**Paper V, Figure 2 A-D; Figure S1**) had a similar morphology to the megalomeronts found during infections of hPARUS1, hPHSIB1 and hPHYBOR04 parasite lineages (**Paper I**, (Ilgūnas et al., 2019)), whereas the megalomeronts found in parasites infections of the lineages hCWT4 and hWW2 were more similar to each other (**Paper V, Figure 2 E-I, T-X; Figures S2, S4**) than to the other parasites of *H. majoris*. The megalomeronts morphology of the same lineage of parasites of *H. majoris* did not differ in different host species (e. g. in

Cyanistes caeruleus Linnaeus, 1758 (Passeriformes: Paridae) and *Parus major* Linnaeus, 1758 (Passeriformes: Paridae), **Paper V**, **Figure 1 J-N, Figure S3** for hPARUS1), or in different seasons (**Paper V**, **Figure 1 J-N, Figure S3** and (Ilgūnas et al., 2019) for hPARUS1). Megalomeronts of *H. majoris* have more morphological similarities across the different lineages (interconnected cytomeres), than with any previously reported megalomeront of other parasite species (Farmer, 1964; Miltgen et al., 1981; Atkinson et al., 1986; Groff et al., 2019; Himmel et al., 2019, 2021; Ilgūnas et al., 2019, 2022; Ortiz-Catedral et al., 2019). This study shows that the morphology of megalomeronts is parasite species-specific for *H. majoris, H. pastoris* and *H. hirundinis*.

The megalomeronts were found at different stages of development (very young, developing, mature, and ruptured) in *H. majoris, H. pastoris* and *H. hirundinis* parasites within host individuals, indicating asynchronous development of the megalomeronts within the host (**Papers I, II, IV, V**). Parasites of *H. pastoris* further exhibit asynchronous development within its megalomeronts with different cytomeres development (**Paper II, Figure 3 I-L, Figure 4 A-H**). Further studies are needed to support this hypothesis.

The brain was found affected for the first time by *Haemoproteus* parasites, with one megalomeront found in one individual infected with *H. pastoris* (hLAMPUR01) (**Paper II, Figure 3 A-D**) and in two individuals infected with *H. majoris* (co-infection of several lineages of *H. majoris*; **Paper V, Figure 3 A, C**). The big megalomeront of *H. majoris* only had eosinophilic staining (**Paper V, Figure 3 A**), with no mature merozoites as observed in the megalomeront found in *H. pastoris* (**Paper II, Figure 3 A-D**), pointing to a stop in the megalomeront development of *H. majoris*. The other found megalomeronts of *H. pastoris* were seen at different stages of development – from developing (without or with clear cytomeres, **Paper II, Figure 3 E-L, Figure 4 A-H**) to mature (with mature merozoites, **Paper II, Figure A-D, Figure 4 I-L**) to ruptured (**Paper II, Figure 3 M-P, Figure 4 M-P, Figure 5**). The biggest megalomeronts accounted for over 600 μm and 800 μm in largest diameter; they were seen in the intestine and pancreas, respectively (**Paper II, Figure 4, I-P**), while *H. handai* (syn. *H. desseri*) Maqsood, 1943 reached 900 μm longitudinally (Miltgen et al., 1981) and clusters of *H. minutus* megalomeronts reached 800 μm (Ortiz-Catedral et al., 2019). However, no very young megalomeronts (around 10 μm) such as the ones found in *H. majoris* lineages hCCF5, hCWT4, hPARUS1 and hPHSIB1 infections (**Paper V, Figure 2 D, I, N, S, Figure 3 B-D, Figures S3, S5**) were reported in either *H. pastoris* (**Paper II**), *H. hirundinis* (**Paper IV**) or other *Haemoproteus* parasite species (Miltgen et al., 1981; Atkinson et al., 1986;

Atkinson et al., 1988). The size megalomeronts can reach seemed more related to its stage of development (very young, developing, mature, ruptured) than to the species or the site of development.

Megalomeronts of *H. majoris* were more commonly found in the kidneys (in 13 out of 43 investigated birds) and the gizzard (in 10 out of 43) of the hosts (**Paper I; Paper V Table 1, Table S2**), with variations between lineages of infection. Exo-erythrocytic stages were found more commonly in the lungs, intestine and gizzard in birds infected with parasites of the lineage hCCF5 (**Paper V Table 1, Figure 2 A-D, Figure S1**); in the intestine and gizzard in hCWT4 infections (**Paper V Table 1, Figure 1 E-I, Figure S2**) and in the kidneys (and the lungs) in hPARUS1 infections (**Paper V Table 1, Figure 2 J-N, Figure S3**, (Ilgūnas et al., 2019)). Only two individuals were found with megalomeronts in birds infected with parasites of the lineages hPHSIB1 (**Paper I, Paper V Table 1, Figure 2 O-S**) and hWW2 (**Paper V Table 1, Figure 2 T-X, Figure S4**), with the kidneys being most affected in hPHSIB1 infections and the gizzard in hWW2 infections. *Haemoproteus minutus* and *H. asymmetricus*, two closely related species recently distinguished (Valkiūnas et al., 2021), developed megalomeronts in the cardiac muscles, gizzard, and lungs (Himmel et al., 2019; Ortiz-Catedral et al., 2019). These findings highlight how closely related lineages of the same parasite species and closely related species might develop similarly in organs of their hosts. Further studies are needed to test this hypothesis.

The number of organs found affected by megalomeronts of *H. majoris* (lineage hPARUS1) was greater in spring (five organs in two individuals) than in autumn (two organs in five individuals) (**Paper V Table 1**). To survive in avian host during unfavourable seasons for transmission (winter in the Palearctic), it would be beneficial for the parasite to adapt for persistence at low intensity in organs so that its influence on hosts health is reduced and probability for the host to survive increases. This study supports this assumption which would be interesting to future research.

Tissue stages of *H. hirundinis* were found only in the pectoral muscles of *D. urbicum* (**Paper IV**), while the tissue stages of *H. pastoris* were found in a total of nine organs (brain, lungs, trachea, kidneys, spleen, oesophagus, pancreas, intestine, and gizzard) in four examined *S. vulgaris* (**Paper II Table 1**). This study is the biggest report of organs affected by exo-erythrocytic stages of *Haemoproteus* parasites, as previous reports usually found the stages in one to four organs (Farmer, 1964; Atkinson et al., 1986; Groff et al., 2019; Himmel et al., 2019, 2021; Ilgūnas et al., 2019, 2022; Ortiz-Catedral et al., 2019). However, tissue stages of *H. majoris* were found in ten organs across

all 45 investigated individuals (**Paper I; Paper V Table 1**, (Ilgūnas et al., 2019)). This might be related to the specificity of the parasites.

The available data show that *H. pastoris*, a more specialist parasite species (mainly found in *S. vulgaris* and few records in other species of the Sturnidae), affected several organs in each positive bird with exo-erythrocytic stages (**Paper II Table 1**); while *H. majoris*, a generalist parasite species (in birds of different families (Nilsson et al., 2016)), affected mostly fewer organs per individual (**Paper V Table 1**). Additionally, *H. hirundinis*, with the two lineages hDELURB1 and hDELURB2, was found in three host species belonging to the Hirundinidae (*D. urbicum, Hirundo rustica* Linnaeus, 1758 (Passeriformes: Hirundinidae) and *Riparia riparia* Linnaeus, 1758 (Passeriformes: Hirundinidae), MalAvi database (Bensch et al., 2009)). However, it is currently unknown if parasites of the lineage hDELURB1 also develop only megalomeronts in the pectoral muscles of their host just as the parasites of the lineage hDELURB2, considering that lineages infections of *H. majoris* (**Papers I, V**, (Ilgūnas et al., 2019)) or that closely related species *H. minutus* and *H. asymmetricus* (Himmel et al., 2019; Ortiz-Catedral et al., 2019) develop similarly. A host-parasite adaptation may have developed. For better understanding pathologies caused by different infections, it would be important to investigate if exo-erythrocytic development is different in other specialist and generalist of *Haemoproteus* parasites, and if exo-erythrocytic development is more lineage-dependent.

3.2. Meronts development in species of *Haemoproteus*: *Haemoproteus attenuatus*, *H. dumbbellus* and *Haemoproteus* spp.

Megalomeronts were not found during natural infections of *H. attenuatus* (lineage hROBIN1) and *H. dumbbellus* (hEMCIR01) in their avian hosts *Erithacus rubecula* Linnaeus, 1758 (Passeriformes: Muscicapidae) and *Emberiza citrinella* Linnaeus, 1758 (Passeriformes: Emberizidae), respectively. Only meronts were observed. The meronts of *H. attenuatus* (hROBIN1) were found in the lungs (in six out of seven individuals in **Paper III**; (Iezhova, 1994)), whereas meronts of *H. dumbbellus* (hEMCIR01) developed in six organs (brain, heart, lungs, liver, gizzard, and leg muscle, in five out of six individuals, **Paper IV Table 1, Figure 4**); this species was described in this study.

The meronts morphology was different in these two species of parasite. Meronts developing in the lungs of *E. citrinella* most often followed the capillaries (**Paper IV Figure 4**), were small (up to 44 μm), elongated, and sometimes clustered together, forming groups (**Paper IV Figure 4**). Meronts

of *H. attenuatus* were bigger (up to 94 μm) and were found aggregating in the capillaries of the lungs (**Paper III Figure 3**).

Two *Fringilla coelebs* Linnaeus, 1758 (Passeriformes: Fringillidae) individuals investigated for *H. majoris* infection were found to have co-infections with other species of *Haemoproteus* and were positive for meronts in the lungs (**Paper V Figure 3 G-I**). Small meronts similar to those of the *H. dumbbellus* (**Paper IV Figure 3**) were found in the co-infection with *H. fringillae* (Labbé, 1894) **(Paper V Figure 3 G)**, while bigger meronts were found in the co-infection with several species of *Haemoproteus* **(Paper V Figure 3 H, I**), making it impossible to correctly determine their species identity without specific CISH probes.

It is important to note that most investigated *Haemoproteus* parasites have been reported to develop either only megalomeronts (**Papers I, II, IV, V** (Farmer, 1964; Atkinson et al., 1986; Groff et al., 2019; Himmel et al., 2019, 2021; Ilgūnas et al., 2019, 2022; Ortiz-Catedral et al., 2019)) or only meronts (**Papers III, IV, V**, (Iezhova, 1994)), further highlighting that exo-erythrocytic development likely is parasite species-specific, but not a parasite-genus characteristic. Further studies are needed to accumulate more information about this issue.

3.3. Can the intensity of parasitemia be a reliable indicator of the presence of exo-erythrocytic stages of *Haemoproteus* parasites?

Investigated host individuals with *H. pastoris* infections had 1 % to 26 % intensity of parasitemia, and megalomeronts were found in all but the individual with 10 % intensity of parasitemia (**Paper II Table 1**). *Haemoproteus majoris* had intensity of parasitemia ranging from 0.01 % to 6.50 % (**Paper I; Paper V Table 1, Table S1**) but megalomeronts were not found in several of the individuals, even if the intensity of parasitemia was similar (**Paper I; Paper V Table S1**). Similar results were also observed in infections of *H. hirundinis*, where intensity of parasitemia was between 0.04 % and 0.53 % and where megalomeronts were found, or not in individuals with similar intensities of parasitemia (**Paper IV Table 1**).

The six *E. rubecula* individuals positive with meronts had intensities of parasitemia between 0.80 % and 26.5 % during *H. attenuatus* infections. The individual negative for tissue stages had 0.95 % intensity of parasitemia. Similarly, *E. citrinella* individuals with meronts of *H. dumbbellus* had intensity of parasitemia between 0.69 % to 1.04 %, while the individual without tissue stages had an intensity of parasitemia of 0.35 %.

This shows that the intensity of parasitemia cannot directly be related to the presence of exo-erythrocytic stages in the host. This parameter is not informative enough when selecting individuals for exo-erythrocytic stages research.

3.4. The importance of chromogenic *in situ* hybridization in research of avian haemosporidian parasites exo-erythrocytic development

The CISH diagnostic method (Dinhopl et al., 2011; Himmel et al., 2019) confirmed the infections of *Haemoproteus* in studied birds with the visualisation of specific CISH signals in blood (confirming the presence of gametocytes) as well as the generic identity of the found exo-erythrocytic stages of *H. majoris* (**Paper V**), *H. hirundinis*, *H. dumbbellus* (**Paper IV**), *H. pastoris* (**unpublished**), *H. attenuatus* (**unpublished**) and other *Haemoproteus* spp. present during co-infections (**Paper V**). The signals obtained from CISH were deep purple in young and developing exo-erythrocytic stages compared to mature stages with little CISH signals when merozoites were developed, reflecting the presence of *Haemoproteus* parasites RNA (**Papers IV, V**, (Himmel et al., 2019)).

Due to their specificity in morphology per parasite species, newly discovered megalomeronts were different from previously observed records (Miltgen et al., 1981; Atkinson et al., 1988; Groff et al., 2019; Himmel et al., 2019), and CISH was essential to confirm megalomeronts of different morphologies and locations (**Papers IV, V, unpublished**). The CISH method also helped to recognize the small stages of *Haemoproteus* parasites, such as the meronts of *H. dumbbellus* (**Paper IV Figure 4**) and very young and small megalomeronts of *H. majoris* (**Paper V Figures 2 D, I, N, S, Figure 3 B-D, Figure S3**). However, the Haemo18*S* probe is *Haemoproteus* genus specific (Himmel et al., 2019); it does not discriminate between the different species nor lineages of *Haemoproteus* parasites. Signals are observed for different *Haemoproteus* parasites present in co-infections, such as seen in co-infection of *H. majoris* with other *Haemoproteus* species (**Paper V Figure 3 G-I**). Species-specific probes would be necessary to resolve co-infections, which are common in wildlife.

3.5. Can molecular phylogenies based of partial cytochrome *b* gene predict patterns of exo-erythrocytic development in *Haemoproteus* parasites?

Over 1900 *cyt b* lineages were reported for parasites of the genus *Haemoproteus* (MalAvi database, accessed in 2023 (Bensch et al., 2009)) and 178 species were described using information about their gametocytes and host cells (**Paper IV**, (Valkiūnas and Iezhova, 2022)). Among these, exo-erythrocytic stages are known for 24 records of parasites (21 species, 15 lineages, but nine species are not characterized molecularly; **Papers I-V**, (Valkiūnas and Iezhova, 2017; Groff et al., 2019; Himmel et al., 2019, 2021; Ilgūnas et al., 2019; Ortiz-Catedral et al., 2019; Hernández-Lara et al., 2021)).

Exo-erythrocytic development of *H. majoris* have been investigated for six closely related lineages (**Papers I, V**, (Ilgūnas et al., 2019)) and only megalomeronts of similar morphology were found (**Paper I Figures 3, 4; Paper V Figures 2, 3, Figures S1-S5**; (Ilgūnas et al., 2019)). Importantly, all these lineages grouped together in a phylogeny based on the partial *cyt b* gene sequences (**Paper I, Figure 2**). This indicates a common morphology per parasite species for which the lineages are closely related to each other. *Haemoproteus pastoris* (**Paper II**) and *H. hirundinis* (**Paper IV**) differ in morphology of their megalomeronts and are neither closely related to *H. majoris* nor do they cluster together as *H. majoris* lineages do (**Papers I, IV**). This shows parasite species-specific morphology of the megalomeronts which coincide with the morphospecies. Based on this observation, the parasite of the lineage hEMSPO03 closely related to *H. majoris* lineages (hCCF5, hCWT4, hPARUS1, hPHSIB1, hPHYBOR04 and hWW2) was also found to develop megalomeronts of similar morphology in the muscular layer of the gizzard as *H. majoris* and was suggested to belongs to *H. majoris* (Himmel et al., 2021). In other words, the available data suggest that *Haemoproteus* species identification might be possible on tissue stages and worth more attention in the parasite taxonomy.

Haemoproteus velans Coatney and Roudabush, 1937, *Haemoproteus syrnii* (Mayer, 1910) and *Haemoproteus sacharovi* Novy and MacNeal, 1904 were found to develop megalomeronts only in the muscles of their hosts (pectoral muscles or smooth muscle of the gizzard) (Farmer, 1964; Groff et al., 2019; Ilgūnas et al., 2022) and cluster together in a phylogeny of partial *cyt b* sequences (**Paper II, Figure 2**). This indicates that closely related parasites can develop in the same organ. However, in the phylogenetic analysis, these three species do not cluster with *H. hirundinis* (**Paper IV**) which also develop megalomeronts in the pectoral muscles but is more distantly related. No other closely related parasite species to *H. hirundinis*

have been investigated for their exo-erythrocytic stages (**Paper IV, Figure 3**), so it is unknown if only this parasite species would develop in the muscles or if other closely related *Haemoproteus* parasite would also develop in the muscles. In other words, some species of *Haemoproteus* are very specific in their site of development, but this feature does not restrict species to be closely related.

Haemoproteus minutus (hTURDUS2) and *H. asymmetricus* (hTUPHI01) were recently distinguished as different species (Valkiūnas et al., 2021), and are closely related in phylogenetic analyses (**Paper II Figure 2; Paper IV Figure 3**). Both parasite species also develop megalomeronts of similar morphology which grouped tightly in the organs, forming big clusters (Himmel et al., 2019; Ortiz-Catedral et al., 2019). Both studies found the megalomeronts in non-adaptive host (parrots (Himmel et al., 2019; Ortiz-Catedral et al., 2019)), whereas their natural host (thrushes) have not been investigated for exo-erythrocytic stages of these parasite species. It would be important to investigate natural hosts for tissue stages and see if the parasites develop similarly in non-adaptive and natural hosts, or if infections in non-adaptive host develop in a different, abortive manner.

The site of development of *H. majoris* megalomeronts showed variation between the lineages' infections, with the kidneys and gizzard more commonly affected throughout the lineages. Similarly, hosts with infection of *H. hirundinis* lineage hDELURB1 can be hypothesized to have megalomeronts developing in their pectoral muscles with similar morphology in the same manner as the closely related *H. hirundinis* lineage hDELURB2 (**Paper IV**). This hypothesis, and other parasite species with closely related lineages (*Haemoproteus tartakovskyi* Valkiūnas, 1986*, Haemoproteus lanii* Mello, 1936*, Haemoproteus balmorali* Peirce, 1984), could be tested to better understand the predictability of phylogenies based on partial *cyt b* gene in research of exo-erythrocytic development.

The lineage hEMCIR01 of *H. dumbbellus* is phylogenetically closely related to hROBIN1 of *H. attenuatus* (**Paper IV**, **Figure 3**), and both parasites developed meronts (**Paper III, Figures 2, 3**; (Iezhova, 1994); **Paper IV, Figure 4)**, but not megalomeronts. *Haemoproteus passeris* Kruse, 1890 was reported to develop meronts (Peirce, 1976), and all three parasite species grouped together in phylogenetic analysis (**Papers I, II, Figure 2; Paper III, Figure 1, Paper IV, Figure 3**). This cluster of species of *Haemoproteus* could be developing only meronts while most other parasite species would develop only megalomeronts. *Haemoproteus fringillae* is also found in this cluster and was hypothesized to develop meronts. The meronts found in the co-infections of *H. majoris* with other *Haemoproteus* species were speculated to be *H.*

fringillae or *Haemoproteus* spp. depending on the individual (**Paper V, Figure 3**), as *H. majoris* developed only megalomeronts in single infections (**Papers I, V**, Ilgūnas et al. 2019). This might indicate a possible pattern of meront development; however, more investigation from closely and distantly related species are necessary to further test this hypothesis.

CONCLUSIONS

1. The morphology and site of development in birds differed between megalomeronts of different parasite species (*H. majoris, H. pastoris* and *H. hirundinis*), and their size varied from less than 10 μm (very young) up to 800 μm.
2. Meronts developed mainly in the lungs' blood vessels of their host and did not exceed 100 μm in parasite of *Haemoproteus attenuatus, H. dumbbellus* and *Haemoproteus* spp. (not identified to the species level).
3. None of the investigated *Haemoproteus* parasites were found to develop both meronts and megalomeronts.
4. *Haemoproteus* parasites developed megalomeronts and meronts in the brain, indicating the existence of cerebral haemoproteosis.
5. The intensity of parasitemia was not associated with exo-erythrocytic stages and should not be considered as a reliable indicator of individuals most suitable for *Haemoproteus* parasites' exo-erythrocytic stages investigations.
6. The chromogenic *in situ* hybridization method was essential to confirm the generic identity of tissue stages in *Haemoproteus* parasites and helped to locate small or irregularly shaped exo-erythrocytic stages.
7. Partial cytochrome *b* gene phylogenetic analysis did not reveal patterns of exo-erythrocytic development regarding the location in organs of *Haemoproteus* parasites. However, it points to a cluster of parasite species developing only meronts as well as to similar morphologies of exo-erythrocytic stages in closely related lineages of the same parasite species. This should be considered in future taxonomic research.

SANTRAUKA

MOKSLINĖ PROBLEMA

Hemosporidiniai parazitai (Apicomplexa, Haemosporida) yra roplių, paukščių ir žinduolių endoparazitai (Garnham, 1966; Valkiūnas, 2005; Telford Jr., 2009). Paukščių hemosporidijos aptinkamos visame pasaulyje, išskyrus Antarktidą (Clark et al., 2014). Jie skirstomi į keturias šeimas – Plasmodiidae, Garniidae, Haemoproteidae ir Leucocytozoidae (Valkiūnas, 2005) – o jų genetinės linijos užfiksuotos daugiau kaip 2195 paukščių rūšyse (MalAvi duomenų bazė, (Bensch et al., 2009)). Šie obligatiniai heterokseniniai patogenai vystosi dviejuose šeimininkuose: paukščių šeimininkas, kuriame vyksta nelytinis dauginimasis ir vystosi gametocitai (invazinė pernešėjui stadija), ir dvisparnis pernešėjas, kuriame vyksta lytinis dauginimasis ir vystosi sporozoitai (invazinė paukščių šeimininkui stadija) (Valkiūnas, 2005; Valkiūnas ir Atkinson, 2020). Išskirti du *Haemoproteus* Kruse, 1890 genties porūšiai – *Haemoproteus* Kruse, 1890 ir *Parahaemoproteus* Bennett, Garnham ir Fallis, 1965. *Haemoproteus* rūšių parazitus perneša briedmusės (Diptera: Hippoboscidae), o *Parahaemoproteus* rūšių atstovus – *Culicoides* Latreille, 1809 (Diptera: Ceratopogonidae) smulkieji mašalai (Valkiūnas, 2005; Bukauskaitė et al., 2019; Valkiūnas ir Atkinson, 2020). Pastarasis porūšis atstovauja daugumai aprašytų hemosporidijų rūšių ir genetinių linijų, taip pat jis yra šio tyrimo objektas.

Haemoproteus ir kiti hemosporidiniai parazitai savo paukščių šeimininkus užkrečia tik per sporozoito stadiją, infekuotų pernešėjų įkandimų metu ir patenka į periferinę kraujotaką. Parazitas prasiskverbia į įvairias šeimininko ląsteles, virsta vienaląsčiais trofozitais, o vėliau – egzoeritrocitinėmis stadijomis (merontais ir (arba) megalomerontais). Pastariesiems subrendus, šeimininko ląstelė plyšta ir merozoitai išsilaisvina, įsiskverbia į eritrocitus ir virsta gametocitais arba prasiskverbia į kitų audinių ląsteles ir tęsia nelytinį dauginimąsi organuose. Iš gametocitų vektoriuose susidaro gametos (Valkiūnas, 2005; Valkiūnas ir Atkinson, 2020).

Naujų *Haemoproteus* parazitų rūšių apibūdinimas šiuo metu grindžiamas gametocitų morfologiniais požymiais ir jų įtaka šeimininko ląstelėms (Valkiūnas, 2005; Valkiūnas ir Iezhova, 2022). Taip pat galima remtis ribotais eksperimentiniais duomenimis apie stuburinių šeimininkų specifiškumą (Bukauskaitė et al., 2019; Chagas et al., 2019). Genetinis parazito apibūdinimas grindžiamas daline mitochondrinio citochromo *b* (*cyt b*) geno seka, apibrėžiama 478 bp brūkšninio kodavimo (*barkodingo*) sritimi ((Bensch et al., 2000; Hellgren et al., 2004), MalAvi duomenų baze (Bensch et al.,

2009)) ir vadinama linija. Nauja linija apibrėžiama, kai tiriama seka bent viena bazių pora skiriasi brūkšninio kodavimo regione nuo bet kurios kitos žinomos sekos (Bensch et al., 2009).

Haemoproteus parazitai, anksčiau laikyti santykinai nekenksmingomais savo paukščių šeimininkams, palyginti su artimai gimininga is *Plasmodium* Marchiafava ir Celli, 1885 ir *Leucocytozoon* Berestneff, 1904 parazitais (Bennett et al., 1993), dažnai buvo ignoruojamos parazitologijoje ir veterinarinėje medicinoje, o jų gyvenimo ciklas liektebėra nežinomas daugumos aprašytų rūšių ir linijų atvejais. Iki šiol aprašytos 178 *Haemoproteus* rūšys (Valkiūnas ir Iezhova, 2022; Duc et al., 2023), o identifikuotos 1904 linijos. Tačiau aprašyta tik 161 linija priskirta atitinkamoms parazitų rūšims ir tik 78 aprašytoms rūšims yra priskirtos genetinės linijos (MalAvi duomenų bazė (Bensch et al., 2009)).

Daugybė parazitų aprašymų datuojami iki PGR protokolų sukūrimo paukščių hemosporidijų tyrimuose, jiems trūksta molekulinio apibūdinimo (Aragão, 1908; Farmer, 1964; Baker, 1966; Khan ir Fallis, 1969; Peirce, 1976; Ahmedt ir Mohammed, 1977; Miltgen et al., 1981; Atkinson et al. 1986; Atkinson ir Forrester, 1987; Atkinson et al., 1988; Earle et al., 1993; Cardona et al., 2002; Paperna ir Gill, 2003; Peirce et al., 2004), o molekulinių tyrimų metu kraujo tepinėliai dažniausiai nebūna analizuojami (Clark et al. (2014) ir Bernotienė et al. (2016), todėl sparčiai didėja atotrūkis tarp šių parazitų rūšių taksonominės ir molekulinės įvairovės. Iki 2019 m. buvo žinomos 15 *Haemoproteus* rūšių egzoeritrocitinės stadijos (merontai ir (arba) megalomerontai) (Valkiūnas ir Iezhova, 2017), daugiausia iš seniai publikuotų tyrimų. Tikroji šių parazitų audinių stadijų įvairovė ir specifiškumas lieka nežinomi, o filogenetinė analizė negalėjo paaiškinti jų egzoeritrocitinių stadijų vystymosi ypatumų, nes trūko informacijos apie daugumą aprašytų rūšių.

Neseniai Ortiz-Catedral et al. (2019) ir Himmel et al. (2019) nustatė *Haemoproteus* (*Parahaemoproteus*) infekcijas negyvose papūgose, daugybė megalomerontų rasta susikaupusių širdyje ir kituose organuose. Šiuose dviejuose tyrimuose rastos stadijos buvo panašios morfologijos, o audinių stadijų *Haemoproteus* kilmė buvo įrodyta sukūrus ir pritaikius genčiai būdingą molekulinę diagnostiką chromogenine *in situ* hibridizacijąa (CISH) (Himmel et al., 2019). Ortiz-Catedral et al. (2019) pranešė apie 12 rūšių papūgų, kurios paprastai neserga šiomis infekcijomis laukinėje gamtoje, gaišimą. Parazitai buvo molekuliniu būdu identifikuoti kaip *Haemoproteus minutus* Valkiūnas ir Iezhova, 1992 ir *H. asymmetricus* Valkiūnas, Ilgūnas, Bukauskaitė, Duc ir Iezhova, 2021, įprasti strazdų (Passeriformes: Turdidae) parazitai. Remiantis šiais duomenimis, iškilo klausimas, ar hemoproteozės ir

audinių stadijų ypatumai, apie kuriuos pranešta neįprastuose paukščių šeimininkuose, yra susiję su parazito vystymusi neadaptuotuose šeimininkuose ir reiškia galimas abortyvines infekcijas, ar rasti megalomerontai yra parazito rūšies požymiai. Vis dar neaišku, ar egzoeritrocitinio vystymosi ciklas yra parazito rūšies, ar šeimininko rūšies, ar abiejų rūšių požymis. Kalbant apie paukščių hemoproteozę, taip pat reikėtų apsvarstyti šiuos su sveikata susijusius klausimus: i) kiek šios infekcijos yra žalingos paukščiams, ii) kaip ir kokiuose organuose jos vystosi ir iii) ar generalistiniai ir specializuoti parazitai vystosi panašiai?

Tiriant audinių stadijas, reikia paimti šeimininko vidaus organų mėginius iš natūraliai nugaišusių individų arba po eutanazijos. Parazitemija yra geras paukščių infekcijos indikatorius (Valkiūnas, 2005; Valkiūnas ir Atkinson, 2020). Užsikrėtusius individus paprastai galima lengvai nustatyti ir apskaičiuoti parazitemijos intensyvumą mikroskopuojant kraujo tepinėlius (Godfrey et al., 1987;Valkiūnas ir Iezhova, 2022). Tačiau lieka neaišku, kaip parazitemijos intensyvumas susijęs su šeimininko egzoeritrocitinio vystymosi intensyvumu ir dėsningumais. Maža parazitemija gali reikšti du skirtingus įvykius: i) egzoeritrocitinės stadijos vis dar vystosi ir tik kelios iš jų subrendo ir išleido merozoitus, kurie įsiskverbė į eritrocitus ir išsivystė į gametocitus; arba ii) egzoeritrocitinės stadijos pratrūko prieš imant mėginius, ir organuose likusių audinių stadijų lieka nedaug, todėl sunku jas stebėti. Didelė parazitemija gali būti dėl to, kad i) dauguma egzoeritrocitinių stadijų buvo subrendusios ir plyšusios, tačiau jų vis dar gali būti organuose, arba ii) visos audinių stadijos neseniai plyšo ir išlaisvino merozoitus, todėl organuose jas būtų sunku aptikti. Šiems klausimams išspręsti reikalingi papildomi tyrimai ir eksperimentai, susiję su parazitemijos ir egzoeritrocitinių stadijų vystymosi intensyvumo santykiu.

Dauguma paskelbtų pranešimų apie hemoproteidų audinių stadijas yra pavieniai stebėjimai, kuriuose pateikiama informacija tik apie vieną infekcijos vystymosi tašką – šeimininko mirtį, atitinkančią parazito vystymosi sustojimą. Yra tik du eksperimentiniai tyrimai (Atkinson et al., 1986; Atkinson et al., 1988), kuriuose *Haemoproteus* parazitų egzoeritrocitinis vystymasis buvo stebimas viso užsikrėtimo metu, pateikiant duomenis apie vienos rūšies šeimininko, užsikrėtusio vienos rūšies parazitu, vystymosi modelį laike. Kitų eksperimentų šia tema nėra, o tai atskleidžia *Haemoproteus* rūšių egzoeritrocitinio vystymosi eksperimentinių tyrimų sunkumus, nes reikalingos sporozoitų sukeltos infekcijos, o tai yra sudėtinga pasiekti, ypač kalbant apie laukinių paukščių parazitus (Bukauskaitė et al., 2019a). Infekuoto paukščių kraujo perpylimas kitam neinfekuotam šeimininkui yra neefektyvus ir negali būti naudojamas *Haemoproteus* rūšių eksperimentiniuose tyrimuose,

o tai akivaizdžiai skiriasi nuo paukščių *Plasmodium* parazitų eksperimentinių tyrimų (Garnham, 1966). Visa tai padidina lauko stebėjimų vertę šioje parazitologijos šakoje, ypač tiriant *Haemoproteus* rūšių parazitų egzoeritrocitines stadijas, kurios yra prieinamos imant natūraliai užsikrėtusių laukinių paukščių mėginius, ir šia galimybe buvo pasinaudota šiame tyrime.

TYRIMO TIKSLAS IR PAGRINDINIAI UŽDAVINIAI

Šio tyrimo tikslas buvo praplėsti žinias apie paukščių hemosporidinių parazitų, priklausančių genčiai *Haemoproteus* (pogentė *Parahaemoproteus*) (Haemosporida, Haemoproteidae), egzoeritrocitinį vystymąsi, naudojant natūraliai užsikrėtusius laukinius paukščius.

Šiam tikslui pasiekti buvo iškeltos šios užduotys:

1. Ištirti ir atlikti palyginamąsias *Haemoproteus majoris*, *Haemoproteus pastoris*, *Haemoproteus hirundinis*, *Haemoproteus attenuatus*, ir *Haemoproteus dumbbellus* egzoeritrocitines stadijas šių parazitų stuburiniuoe šeimininkuose.
2. Nustatyti parazitemijos intensyvumo, kaip parametro individų atrinkimui egzoeritrocitinių stadijų vystymosi natūralių *Haemoproteus* infekcijų metu tinkamumą.
3. Pritaikyti choromogeninės *in situ* hibridizacijos metodą siekiant patvirtinti parazito egzoeritrocinių stadijų buvimą bei gentį, kuriai priskiriamos šios stadijos.
4. Nustatyti ar turimi duomenys apie parazitų rūšis bei filogenetinė analizė paremta daline *cyt b* geno seka gali būti naudojama kaip indikatorius apie *Haemoproteus* parazitų egzoeritrcotinių stadijų morfologiją, jų lokalizaciją organuose ir vystymosi dėsningumus natūraliai užsikrėtusiuose paukščiuose.

GINAMIEJI TEIGINIAI

1. *Haemoproteus majoris*, *Haemoproteus pastoris* ir *Haemoproteus hirundinis* vysto skirtingos morfologijos megalomerontus skirtinguose skirtinguose šeimininko organuose.
2. *Haemoproteus attenuatus* ir *H. dumbbellus* parazitai vysto merontus plaučiuose; megalomerontai tirtuose individuose nerasti.
3. *Haemoproteus* parazitai vysto egzoeritrocitines stadijas užkrėstų paukščių smegenyse ir šiame darbe pirmą kartą aprašyta cerebralinė *H. pastoris, H. majoris* ir *H. dumbbellus* haemoproteozė.

4. Parazitemijos intensyvymas nėra susijęs su egzoeritrocitinių *H. majoris*, *H. pastoris*, *H. hirundinis*, *H. attenuatus* ir *H. dumbbellus* stadijų vystymusi natūralių infekcijų metu.
5. Chromogeninė *in situ* hibridizacija yra tinkamas metodas mažų *Haemoproteus* egzoeritrocitinių stadijų aptikimui organuose iš šių stadijų įdentifikavimui iki genties
6. Šiuo metu prieinama informacija apie egzoeritrocitines stadijas ir filogenetines analizes paremtas daline *cyt b* geno seka yra nepakankama norint nuspėti *Haemoproteus* genties parazitų vystymąsi šeimininko organuose, tačiau gali būti naudinga dirbant su artimai susijusiomis linijomis.
7. Visų tirtų parazitų rūšių atstovų audinių stadijų morfologija skyrėsi, o tai reiškia, kad ši informacija yra naudinga iš taksonominio požiūrio.

TYRIMŲ NAUJUMAS IR AKTUALUMAS

1. Nustatyta, kad egzeoeritrocitinis parazitų vystymasis yra rūšiai specifiškas. *Haemoproteus majoris*, *H. pastoris* ir *H. hirundinis* parazitai tirtuose individuose vystė tik megalomerontus, o *H. attenuatus* ir *H. dumbbellus* tik merontus.
2. Visi tirti *Haemoproteus* parazitai vystėsi skirtinguose organuose, kas rodo jog reikia ištirti kaip galima daugiau vidaus organų dirbant su dar neištirtais parazitais.
3. Cerebralinė haemoproteozė buvo nustatyta pirmą kartą, tiek merontai tiek megalomerontai buvo rasti smegenyse, kas reiškia anksčiau nežinotos patologijos atradimą.
4. Pirmą kartą rasti labai jauni megalomerontai, priskirti skirtingoms *H. majoris* genetinės linijoms. Jie rasti pavasarį, vasarą ir rudenį, tačiau lieka neaišku ar jie išlieka ir žiemą.
5. Individų atranka parazitų egzoeritrocitiniams tyrimams netūrėtų būti paremta vien parazitemijos intensyvumo parametru.
6. Hemosporidijų egzoeritrocitinių stadijų tyrimuose turėtų būti kombinuojama tiek hematoksilenu-eozinu dažytų histologinių pjūvių analizė (informacijai apie struktūrą, morfologiją), tiek CISH apdorodų pjūvių analizė (mažų, netipinės formos stadijų analizei ir apibūdinimui iki genties).
7. Trys genetinės linijos buvo pirmą kartą priskirtos aprašytoms parazitų rūšims: hEMCIR01 priskirta naujai aprašytai rūšiai *H. dumbbellus*, hDELURB2 priskirta *H. hirundinis,* o hCWT4 priskirta *H. majoris*. Molekulinis parazitų apibūdinimas yra svarbus plečiant *Haemoproteus*

parazitų tyrimų galimybes bei tolimesniems tyrimams susijusiems su parazitų vystymusi ir filogenija.

8. Turima informacija yra nepakankama norint atsakyti į klausimą ar filogenetinė analizė gali padėti nuspėjant *Haemoproteus* genties parazitų egzoeritrocitinį vystymąsi.
9. Artimai susijusios *H. majoris* genetinės linijos išvystė panašias egzoeritrocitines stadijas atitinkamuose šeimininko organuose, kas rodo jog artimai susijųsių linijų infekcijų atvejais filogenija gali padėti nuspėti egzoeritrocitinių stadijų morfologiją ir lokaciją skirtinguose stuburiniuose šeimininkuose.
10. Morfologiniai egzoeritrocitinių stadijų požymiai buvo pastovųs tirtų parazitų rūšių atvejais, dėl ko gali būti naudingas požymis *Haemoproteus* rūšių taksonomijoje.

TRUMPA LITERATŪROS APŽVALGA

Literatūros apžvalgoje glaustai pateikiama informacija apie *Haemoproteus* parazitų tyrimų istoriją, atliktų tyrimų svarbą bei detalesnių tyrimų poreikį apie šių patogenų egzoeritrocitinį vystymąsi.

MEDŽIAGA IR METODAI

Tyrimų metu buvo taikomi įvairūs metodai. Pasirinkta metodika apėmė lauko tyrimus, mikroskopiją ir vaizdų analizę, DNR išskyrimą, PGR amplifikaciją, DNR sekoskaitą ir filogenetines analizes, histologiją ir chromogeninę *in situ* hibridizaciją bei statistinę analizę. Išsamūs tyrimų ir metodų aprašymai pateikiami paskelbtuose originaliuose straipsniuose (**I–V straipsniai**). Toliau pateikiami pagrindinių naudotų tyrimo protokolų trumpi aprašymai.

Tyrimo vietos

Tyrimų medžiaga buvo surinkta 2016 – 2022 m., Labanoro regioniniame parke (55°12’25.77” N, 25°56’26.47” E) (**I straipsnis**) ir Ventės rago ornitologinėje stotyje (55°20’38.93” N, 21°11’34.05” E) (**II, III, IV, V straipsniai**). Mikroskopiniai ir molekuliniai tyrimai bei histologinės procedūros atliktos Gamtos tyrimų centro P. B. Šivickio parazitologijos laboratorijoje, Vilniuje (Lietuva) (https://www.gamtostyrimai.lt) (**I–V straipsniai**) ir Vienos veterinarijos universiteto Patobiologijos katedros Patologijos institute, Vienoje (Austrija) (https://www.vetmeduni.ac.at) (**IV, V straipsniai**). Reprezentaciniai kraujo tepinėliai su gametocitais ir histologinių pjūvių preparatai su egzoeritrocitinėmis stadijomis yra deponuoti Gamtos

tyrimų centre (**I–V straipsniai**) ir Kvynslando muziejuje, Brisbane, Australijoje (**IV, V straipsniai**), ir jie yra prieinami susisiekus su autoriais-korespondentais (**I–V straipsniai**).

Mėginių rinkimas

Kraujo mėginiai buvo imami, pradūrus paukščio brachialinę veną, dalis kraujo panaudota molekuliniams tyrimams, iš kraujo lašo buvo ruošiami kraujo tepinėliai parazitams identifikuoti ir parazitemijai įvertinti (**I–V straipsniai**). Buvo atrinkti paukščiai, užsikrėtę *Haemoproteus* rūšių parazitais, jų organai paimti histologiniams tyrimams (**I–V straipsniai**).

Haemoproteus rūšys buvo identifikuotos ir aprašytos, remiantis standartiniais gametocitų ir šeimininko ląstelių morfologiniais požymiais (Valkiūnas, 2005; Valkiūnas ir Iezhova, 2022). Morfologinė identifikacija buvo patvirtinta DNR sekų informacija (**I–V straipsniai**).

Histologiniai metodai

Histologinei analizei buvo atlikti parafino blokų pjūviai. Jie buvo nudažyti naudojant H&E, siekiant nustatyti egzoeritrocitines stadijas organuose, arba apdoroti naudojant CISH su Haemo18*S* žymeniu, siekiant patvirtinti ir padidinti stadijų aptikimą (**I–V straipsniai**).

Molekulinė analizė

Iš kraujo mėginių arba užšaldytų negyvų paukščių audinių mėginių buvo išskirta DNR (**I–V straipsniai**). Siekiant nustatyti infekciją sukėlusių parazitų linijas, buvo atliktos skirtingos lizdinės PGR. Patikrinta, ar PGR produktų amplifikacijos yra teigiamos, ir jų sekos nuskaitytos 5' ir 3' kryptimis (**I–V straipsniai**). Patikrinus chromatogramas, viengubos infekcijos buvo patvirtintos pagal pavienius nukleotidų pikus, o mišrios infekcijos buvo nustatytos, remiantis dvigubais nukleotidų pikais toje pačioje pozicijoje. Gautos sekos buvo identifikuotos, naudojant BLAST įrankį MalAvi (Bensch et al., 2009) ir NCBI GenBank (National Library of Medicine, Bethesda, Maryland, https://www.ncbi.nlm.nih.gov/genbank/) duomenų bazėse. Visos gautos sekos buvo deponuotos duomenų bazėje GenBank (**I–V straipsniai**). Nustatytų parazitų rūšių *cyt b* geno DNR sekos buvo naudojamos Bajeso filogenetinio medžio skaičiavimams.

Etinis pareiškimas

Visos procedūros su paukščiais buvo atliekamos pagal leidimus ir yra atitinkančios Lietuvos ir Austrijos įstatymus. Su paukščiais susijusias procedūras atliko ir mėginius rinko licencijuoti tyrėjai, visa tai patvirtinta Lietuvos Aplinkos apsaugos agentūros, Vilnius (leidimų numeriai: Leidimai: 2016 05 05 Nr. 23; 2017 04 26 Nr. 23; 2017 06 05 Nr. 33; 2018 04 13 Nr. 24; 2019 04 19 Nr. 23; 2020 04 08 Nr. (26)-A4E-2892 (2020 04 07 Nr. 21); 2021 05 05 Nr. (26)-SR-96 ir 2022 04 28 Nr. (26)-SR-152) (**I–V straipsniai**). Patologijos institute surinktiems mėginiams papildomo etinio patvirtinimo nereikėjo, nes jie buvo surinkti atliekant įprastas diagnostikos paslaugas Vienos Veterinarinės medicinos universitete (**IV straipsnis**).

REZULTATAI IR DISKUSIJA

Visi rezultatai buvo aprašyti ir aptarti paskelbtuose straipsniuose, kuriais paremta ši disertacija (**I–V straipsniai**). Toliau apibendrinami pagrindiniai disertacijos uždavinius atitinkantys rezultatai.

Trijų *Haemoproteus* rūšių megalomeronų vystymasis: *Haemoproteus majoris*, *H. pastoris* ir *H. hirundinis*.

Ieškant *Haemoproteus majoris* egzoeritrocitinių stadijų, ištirti 43 individai, priklausantys 14-kai paukščių rūšių (**I, V straipsniai**). Visuose skirtingomis *H. majoris* linijomis: hCCF5, hCWT4, hPARUS1, hPHSIB1, hPHYBOR04 ir hWW2 užsikrėtusiuose individuose, kuriuose rastos egzoeritrocitinės stadijos, rasti tik megalomerontai (**I, V straipsniai,** (Ilgūnas et al., 2019)). *Haemoproteus pastoris* megalomerontai rasti keturiuose iš penkių *Sturnus vulgaris* Linnaeus, 1758 (Passeriformes: Sturnidae) (**II straipsnis**). Iš šešių *Delichon urbicum* Linnaeus, 1758 (Passeriformes: Hirundinidae) (**IV straipsnis**) megalomerontai rasti tik dviejų individų krūtinės raumenyse, ir lyginant su kitais aprašytais *Haemoproteus* megalomerontais, pastarieji buvo pailgi, o ne apvalūs (**I, II, IV, V straipsniai**, (Groff et al., 2019; Himmel et al., 2019, 2021; Ilgūnas et al., 2019, 2022; Ortiz-Catedral et al., 2019)). Citomerai, rasti megalomerontuose, morfologiškai skyrėsi skirtingų infekcijų atvejais, *H. majoris* – labiau susiję tarpusavyje (**I, V straipsniai**, (Ilgūnas et al., 2019)), *H. pastoris* infekcijos metu – labiau kondensuoti (**II straipsnis**), *H. hirundinis* infekcijos atveju – formavosi žvaigždės forma su išsikišančiais merozoitais (**IV straipsnis**). Kiekvienos iš trijų parazitų rūšių megalomerontai buvo panašios morfologijos, lyginant atitinkamos parazito rūšies stadijas tarp tos pačios rūšies stuburinių šeimininkų individų, bet skyrėsi tarpusavyje,

lyginant skirtingų rūšių parazitus šio tyrimo metu (**I, II, IV, V straipsniai**) ir remiantis literatūros duomenimis (Farmer, 1964; Miltgen et al., 1981; Atkinson et al., 1986; Groff et al., 2019; Himmel et al., 2019, 2021; Ilgūnas et al., 2019, 2022; Ortiz-Catedral et al., 2019).

Haemoproteus majoris hCCF5 megalomerontai (**V straipsnis**) buvo panašios morfologijos į hPARUS1, hPHSIB1 ir hPHYBOR04 infekcijų metu rastus megalomerontus (**I straipsnis**, (Ilgūnas et al., 2019)), o hCWT4 ir hWW2 (**V straipsnis**) megalomerontai buvo panašesni tarpusavyje nei į kitų linijų *H. majoris* megalomerontus. Tos pačios *H. majoris* linijos megalomerontai nesiskyrė tarpusavyje, lyginant jų morfologiją skirtinguose paukščiuose (hPARUS1 megalomerontai *Cyanistes caeruleus* Linnaeus, 1758 (Passeriformes: Paridae) ir *Parus major* Linnaeus, 1758 (Passeriformes: Paridae), **V straipsnis)** ar skirtingu metų laiku (hPARUS1 megalomerontai, **V straipsnis** ir (Ilgūnas et al., 2019)). Skirtingų *H. majoris* linijų megalomerontai yra morfologiškai panašesni tarpusavyje nei į kitų parazitų rūšių megalomerontus, aprašytus anksčiau (Farmer, 1964; Miltgen et al., 1981; Atkinson et al., 1986; Groff et al., 2019; Himmel et al., 2019, 2021; Ilgūnas et al., 2019, 2022; Ortiz-Catedral et al., 2019). Disertacijoje aprašomi darbai rodo, kad *H. majoris, H. pastoris* ir *H. hirundinis* megalomerontų morfologija yra rūšiai specifiška.

Skirtingų subrendimo stadijų megalomerontai (labai jauni, bręstantys, subrendę, pratrūkę) buvo rasti besivystantys paraleliai, kas rodo, jog *H. majoris, H. pastoris* ir (ar) *H. hirundinis* megalomerontai stuburiniame šeimininke vystosi asinchroniškai (**I, II, IV, V straipsniai**). *Haemoproteus pastoris* citomerai megalomerontuose taip pat gali vystytis asinchroniškai (**II straipsnis**). Tolimesni tyrimai yra reikalingi siekiant patikrinti šias hipotezes.

Pirmą kartą buvo rastos *Haemoproteus* egzoeritrocitinės stadijos, besivystančios užkrėstų paukščių smegenyse – viename individe užsikrėtusiame *H. pastoris* (hLAMPUR01) (**II straipsnis**) ir dviejuose užsikrėtusiuose *H. majoris* (kelių *H. majoris* linijų koinfekcijos (**V straipsnis**)). Dideli *H. majoris* megalomerontai nusidažė tik eozinofilu (**V straipsnis**), juose, priešingai nei hLAMPUR01 megalomeronte (**II straipsnis**), nesimatė subrendusių merozoitų, kas indikuoja vystymosi sustojimą. Kiti *H. pastoris* megalomerontai buvo rasti skirtingo subrendimo lygių – nuo bręstančių (be aiškiai matomų citomerų) iki subrendusių (su subrendusiais merozoitais) iki pratrūkusių (**II straipsnis**). Didžiausi megalomerontai buvo iki 600 µm ir 800 µm skersmens ir buvo rasti žarnose ir kasoje (**II straipsnis**), tuo tarpu literatūroje aprašytos *H. handai* (sin. *H. desseri*) Maqsood, 1943 siekė 900 µm (Miltgen et al., 1981), o *H. minutus* megalomerontų sankaupos – 800 µm (Ortiz-Catedral et al., 2019). Labai jauni

megalomerontai, tokie kaip *H. majoris* linijų hCCF5, hCWT4, hPARUS1 ir hPHSIB1 infekcijų metu, nebuvo rasti nei *H. pastoris* (**II straipsnis**), nei *H. hirundinis* (**IV straipsnis**), nei anksčiau aprašytų *Haemoproteus* infekcijų (Miltgen et al., 1981; Atkinson et al., 1986; Atkinson et al., 1988) metu. Megalomerontų dydis labiau priklauso nuo megalomeronto subrendimo lygio (labai jaunas, bręstantis, subrendęs, pratrūkęs) nei nuo parazito rūšies ar užkrėsto organo.

Haemoproteus majoris megalomerontai buvo dažniau randami užkrėstų paukščių inkstuose (13iš 43 individų) ir gūžiuose (10 iš 43 indivividų), su tam tikromis variacijomis tarp genetinių linijų (**I, V straipsniai**). Egzoeritrocitinės stadijos dažniau buvo rastos plaučiuose, žarnose ir gūžiuose hCCF5 infekcijos metu (**V straipsnis**), žarnose ir gūžiuose hCWT4 infekcijos metu (**V straipsnis**), inkstuose ir plaučiuose hPARUS1 infekcijos metu (**V straipsnis**, (Ilgūnas et al., 2019)). Tik dviejuose individuose buvo rasti hPHSIB1 (inkstuose) (**I straipsnis**) ir hWW2 (gūžyje) (**V straipsnis**) megalomerontai. *Haemoproteus minutus* ir *H. asymmetricus* genetiškai artimi parazitai išvystė megalomerontus paukščių širdies raumenyse, gūžiuose ir plaučiuose (Himmel et al., 2019; Ortiz-Catedral et al., 2019). Šie duomenys rodo, kaip genetiškai artimi parazitai gali vystytis panašiuose stuburinių šeimininkų organuose. Reikalingi papildomi tyrimai norint patikrinti šią hipotezę.

Organų, kuriuose vystosi *H. majoris* (hPARUS1 linija) megalomerontai, skaičius pavasarį (5 organai dviejuose individuose) buvo didesnis nei rudenį (du organai penkuose individuose) (**V straipsnis**). Siekiant išgyventi stuburiniame šeimininke pernešimui nepalankiu metu (žiema Palearktikoje), parazitui yra naudinga prisitaikyti prie išlikimo stuburiniame šeimininke žemu intensyvumu, taip sumažinant savo poveikį paukščiui ir padidinant jo ir kartu savo tikimybę išgyventi. Disertacijoje aprašomi tyrimai leidžia kelti tokią hipotezę bei nurodo kryptį tolimesniems tyrimams.

Haemoproteus hirundinis buvo rastas viename organe – *Delichon urbicum* krūtinės raumenyje (**IV straipsnis**), o keturiuose ištirtuose *Sturnus vulgaris* parazitai buvo rasti devyniuose organuose (smegenyse, plaučiuose, trachėjoje, inkstuose, blužnyje, stemplėje, kasoje, žarnose ir gūžyje) (**II straipsnis**). Šis tyrimas atskleidžia didžiausią kiekį organų, paveiktų *Haemoproteus* egzoeritrocitinių stadijų, nes ankstesniuose tyrimuose būdavo randama nuo vieno iki keturių paveiktų organų (Farmer, 1964; Atkinson et al., 1986; Groff et al., 2019; Himmel et al., 2019, 2021; Ilgūnas et al., 2019, 2022; Ortiz-Catedral et al., 2019). *Haemoproteus majoris*, ištyrus 45 individus (**I, V straipsniai**, (Ilgūnas et al., 2019)) parazitai buvo rasti dešimtyje organų. Tai gali būti susiję parazitų specifiškumu.

Publikuoti duomenys byloja, kad *H. pastoris* yra labiau specifiškas parazitas (randamas daugiausia *Sturnus vulgaris* ir keliuose kituose Sturnidae paukščiuose), paveikiantis kelis organus kiekviename užkrėstame paukštyje (**II straipsnis**); *H. majoris* yra generalistinis parazitas (randamas skirtingų šeimų paukščiuose (Nilsson et al., 2016)), paveikiantis dažniausiai mažiau organų kiekviename šeimininke (**V straipsnis**). Taip pat dvi *H. hirundinis* linijos hDELURB1 ir hDELURB2 buvo rastos trijuose stuburiniuose šeimininkuose, priklausančiuose Hirundinidae (*Delichon urbicum, Hirundo rustica* Linnaeus, 1758 (Passeriformes: Hirundinidae) ir *Riparia riparia* Linnaeus, 1758 (Passeriformes: Hirundinidae), MalAvi duomenų bazė (Bensch et al., 2009)). Vis dėlto, nėra žinoma, ar hDELURB1 linijos parazitai vysto megalomerontus šeimininko krūtinės raumenyse kaip tai daro hDELURB2, kaip yra skirtingų *H. majoris* linijų (**I, V straipsniai**, (Ilgūnas et al., 2019)) ar genešikai artimų *H. minutus* ir *H. asymmetricus* (Himmel et al., 2019; Ortiz-Catedral et al., 2019) egzoeritrocitinio vystymosi atvejais. Gali būti, jog išsivystė parazito-šeimininko prisitaikymas. Norint geriau suprasti skirtingų infekcijų sukeliamas patologijas, svarbu ištirti, ar specialistai ir generalistai *Haemoproteus* parazitai organuose vystosi skirtingai ir ar egzoritrocitinis vystymasis priklauso nuo parazito genetinės linijos.

Haemproteus rūšių merontų vystymasis: *Haemoproteus attenuatus*, *H. dumbbellus* ir *Haemoproteus* spp.

Megalomerontai nebuvo rasti natūralių *H. attenuatus* (linija hROBIN1) ir *H. dumbbellus* (hEMCIR01) infekcijų metu jų stuburiniuose šeimininkuose *Erithacus rubecula* Linnaeus, 1758 (Passeriformes: Muscicapidae) (6 iš 7 individų) ar atitinkamai *Emberiza citrinella* Linnaeus, 1758 (Passeriformes: Emberizidae) (5 iš 6 individų). Tik *H. attenuatus* (hROBIN1) merontai buvo rasti plaučiuose (**III straipsnis**, (Iezhova, 1994)), o *H. dumbbellus* (hEMCIR01) merontai vystėsi 6 organuose (smegenyse, širdyje, plaučiuose, kepenyse, gūžyje, kojos raumenyse) (**IV straipsnis**); pastarasis parazitas pirmą kartą aprašytas šio darbo metu.

Merontų morfologija skyrėsi tarp šių dviejų rūšių parazitų. *Emberiza citrinella* plaučiuose rasti merontai buvo kapiliaruose, maži (iki 44 μm), pailgi ir kartais grupavosi kartu (**IV straipsnis**). *Haemoproteus attenuatus* merontai buvo didesni (iki 94 μm) ir kaupėsi plaučių kapiliaruose (**III straipsnis**).

Du *Fringilla coelebs* Linnaeus, 1758 (Passeriformes: Fringillidae) individai buvo ištirti dėl *H. majoris* infekcijos. Šiuose paukščiuose buvo nustatyta koinfekcija su kitų rūšių *Haemoproteus* parazitais, ir abiejuose paukščiuose merontai buvo rasti plaučiuose (**V straipsnis**). Maži merontai,

panašūs į *H. dumbbellus* merontus (**IV straipsnis**), buvo rasti koinfekuojantis su *H. fingillae* (Labbé, 1894), o didesni merontai buvo rasti koinfekcijoje su kelių kitų *Haemoproteus* rūšių parazitais (**V straipsnis**), dėl ko, naudojant CISH žymenis, buvo neįmanoma tiksliai nustatyti, kokiai rūšiai priklausė šios stadijos.

Svarbu pastebėti, kad daugiausia ištirtų *Haemoproteus* rūšių vystė arba tik megalomerontus (**I, II, IV, V straipsniai**, (Farmer, 1964; Atkinson et al., 1986; Groff et al., 2019; Himmel et al., 2019, 2021; Ilgūnas et al., 2019, 2022; Ortiz-Catedral et al., 2019)) arba tik merontus (**III, IV, V straipsniai**, (Iezhova, 1994)), toliau patvirtinant, kad egzoeritrocitinis parazitų vystymasis tikriausiai yra rūšims specifiškas ir gali varijuoti genties lygyje. Norint geriau suprasti šį klausimą, reikia sukaupti daugiau informacijos.

Ar parazitemijos intensyvymas yra patikimas egzoeritrocitinių stadijų buvimo indikatorius *Haemoproteus* infekcijos atveju?

Ištirtos *H. pastoris* infekcijos buvo 1 % – 26 % intensyvumo ir megalomerontai buvo rasti visuose užsikrėtusiuose individuose, išskyrus individą su 10 % parazitemija (**II straipsnis**). *Haemoproteus majoris* parazitemija buvo 0,01 % – 6,50 % (**I, V straipsnis**), bet megalomerontai buvo rasti ne visuose tirtuose individuose, net jei parazitemijos intensyvumas juose buvo panašus (**I, V straipsnis**). Panašūs rezultatai gauti ir *H. hirundinis* infekcijos tyrimų metu, kur parazitemijos intesyvumas buvo 0,04 % – 0,53 % ir megalomerontai buvo rasti ne individuose su panašiu parazitemijos intensyvumu (**IV straipsnis**).

Šešių *H. attenuatus* užsikrėtusių *E. rubecula* individų kuriuose rasti merontai, parazitemija varijavo tarp 0.80 % ir 26.5 %. Individo kuriame merontai nebuvo rasti parazitemija buvo 0.95 %. Panašiai buvo ir *H. dumbbellus* užsikrėtusių *E. citronella* atvejais, kai individuose kuriuose rastos egzoeritrocitinės stadijos parazitemija varijavo tarp 0.69 % to 1.04 %, o individido kuriam šios stadijos nerastos parazitemijos intensyvumas - 0.35 %.

Tai rodo, kad parazitemijos intensyvumas nėra egzoeritrocitinių stadijų indikatorius. Tai nėra tinkamas parametras, kuriuo remiantis galima atrinkti individus egzoeritrocitinių hemosporidinių parazitųstadijų tyrimams.

Chromogeninės *in situ* hibridizacijos svarba paukščių hemosporidijų egzoeritrocitinio vystymosi tyrimuose.

CISH diagnostikos metodu (Dinhopl et al., 2011; Himmel et al., 2019) patvirtintos *Haemoproteus* infekcijos tirtuose paukščiuose, nes kraujyje buvo

matomi CISH signalai (patvirtinantys gametocitų buvimą) bei rastų egzoeritrocitinių stadijų priklausimą *Haemoproteus* genties parazitams: *Haemoproteus majoris* (**V straipsnis**), *H. hirundinis*, *H. dumbbellus* (**IV straipsnis**), *H. pastoris* (**nepulikuota**), *H. attenuatus* (**nepublikuota**) ir kelių kitų *Haemoproteus* rūšių koinfekcijos (**V straipsnis**). CISH signalai, gauti jaunose ir brėstančiose egzoeritrocitinėse stadijose, buvo tamsiai violetiniai, o subrendusiose stadijose jie buvo blyškesni, kas leidžia spėti kiek parazito RNR yra šiose stadijose (kuo tamsesnis signalas, tuo daugiau parazito RNR yra; **IV, V straipsniai**, (Himmel et al., 2019)).

Dėl morfologijos ypatybių, būdingų tik atitinkamoms rūšims, naujai atrasti megalomerontai aiškiai skyrėsi nuo anksčiau aprašytų (Miltgen et al., 1981; Atkinson et al., 1988; Groff et al., 2019; Himmel et al., 2019), o CISH buvo panaudota identifikuojant skirtingus megalomerontus skirtinguose organuose (**IV, V straipsniai** ir **nepublikuota**). CISH metodas buvo pasitelktas ir identifikuojant mažas *Haemoproteus* egzoeritrocitines stadijas, tokias kaip *H. dumbbellus* merontai (**IV straipsnis**) ir labai jaunus *H. majoris* megalomerontus (**V straipsnis**). Vis dėlto, Haemo18*S* žymuo yra specifinis genčiai (Himmel et al., 2019) ir nėra tinkamas atskirti skirtingus *Haemoproteus* genties parazitų merontus ar megalomerontus, kaip buvo *H. majoris* ir *Haemoproteus* spp. koinfekcijos atvejais (**V straipsnis**). Rūšiai specifiški žymenys yra reikalingi norint dirbti su koinfekcijomis, kurios gamtoje dominuoja.

Ar molekulinė filogenija, paremta daline citochromo *b* geno seka, gali nuspėti egzoeritrocitinį *Haemoproteus* parazitų vystymąsi?

Daugiau nei 1900 *Haemoproteus* parazitų genetinių linijų buvo rastos iki šiol (MalAvi duomenų bazė, tikrinta 2023 (Bensch et al., 2009)) ir 178 buvo aprašytos, remiantis jų gametocitų ir paveiktų šeimininko ląstelių morfologija (**IV straipsnis**, (Valkiūnas ir Iezhova, 2022)). Iš visų jų, egzoeritrocitinės stadijos aprašytos 24 atvejais (21 rūšis, 15 genetinių linijų, 9 rūšys neapibūdintos molekuliniais metodais) (**I–V straipsniai**, (Valkiūnas ir Iezhova, 2017; Groff et al., 2019; Himmel et al., 2019, 2021; Ilgūnas et al., 2019; Ortiz-Catedral et al., 2019; Hernández-Lara et al., 2021)).

Egzoeritrocitinis *H. majoris* vystymasis buvo ištirtas šešių šio parazito linijų infekcijų metu (**I, V straipsniai**, (Ilgūnas et al., 2019)) ir buvo rasti tik panašios morfologijos megalomerontai. Svarbu pastebėti, kad visos šios linijos filogenetiniame medyje grupuojasi kartu (**I straipsnis**). Tai rodo, kad genetiškai artimų linijų parazitai formuoja panašios morfologijos struktūras. *Haemoproteus pastoris* (**II straipsnis**) ir *H. hirundinis* (**IV straipsnis**)

formuoja skirtingos morfologijos megalomerontus ir nei viena rūšis nėra artimai susijusi su *H. majoris* ir nesigrupuoja su šio parazito linijomis filogenetiniame medyje (**I, IV straipsniai**). Tai rodo, kad šio parazitų formuojami megalomerontai yra rūšiai specifiškos morfologijos. Remiantis šia prielaida, nustatyta, kad hEMSPO03 yra artimai susijusi su *H. majoris* linijomis (hCCF5, hCWT4, hPARUS1, hPHSIB1, hPHYBOR04 ir hWW2) ir formuoja panašios morfologijos megalomerontus šeimininko gūžyje kaip ir *H. majoris*. Spėjama, kad ši linija taip pat priklauso *H. majoris* parazitui (Himmel et al., 2021). Kitaip tariant, turimi duomenys indikuoja, kad egzoeritrocitinės stadijos yra vertos daugiau dėmesio žvelgiant iš parazitų taksonomijos perspektyvos.

Haemoproteus velans Coatney ir Roudabush, 1937, *Haemoproteus syrnii* (Mayer, 1910) ir *Haemoproteus sacharovi* Novy ir MacNeal, 1904 formuoja megalomerontus tik šeimininko raumenyse (krūtinės, lygiuosiuose arba gūžio raumenyse) (Farmer, 1964; Groff et al., 2019; Ilgūnas et al., 2022) ir šių parazitų genetinės linijos yra filogeniškai artimos (**II straipsnis**). Tai rodo, kad genetiškai artimi parazitai gali vystytis tuose pačiuose šeimininkų organuose. Tačiau nei vienas iš šių parazitų nėra artimas *H. hirundinis* parazitui, kuris taip pat formuoja megalomerontus šeimininko raumenyse (**IV straipsnis**). Joks kitas filogenetiškai artimas *H. hirundinis* parazitas nebuvo ištirtas egzoeritrocitinio vystymosi požiūriu (**IV straipsnis**), todėl nėra žinoma, ar raumenyse vystosi tik šios rūšies ar ir jiems artimų rūšių patogenai. Kitaip tariant, yra *Haemoproteus* rūšių, kurios vystosi tik tam tikrose šeimininko organuose, tačiau tai nebūtinai viena kitai artimos rūšys.

Haemoproteus minutus (hTURDUS2) ir *H. asymmetricus* (hTUPHI01) buvo neseniai išskirtos kaip atskiros parazitų rūšys (Valkiūnas et al., 2021) ir yra filogenetiškai artimos (**II, IV straipsniai**). Abiejų rūšių atstovai formuoja panašios morfologijos susigrupavusius megalomerontus (Himmel et al., 2019; Ortiz-Catedral et al., 2019). Žinoma, kad šie megalomerontai randami tik neprisitaikiusiuose šeimininkuose (papūgose (Himmel et al., 2019; Ortiz-Catedral et al., 2019)), o natūralūs šių parazitų šeimininkai – strazdai – nebuvo ištirti šiuo aspektu. Svarbu ištirti, kaip panašiai šios infekcijos vystosi natūraliuose ir neadaptuotuose šeimininkuose.

Haemoproteus majoris išvystė panašius megalomerontus artimai susijusių linijų skirtingų infekcijų metu (**I, V straipsniai**, (Ilgūnas et al., 2019)). Vieta, kur vystėsi šios struktūros, buvo skirtinga, o inkstai ir gūžys buvo dažniausiai paveikiami organai. *Haemoproteus hirundinis* linija hDELURB1 galimai vysto megalomerontus užkrėstų paukščių krūtinės raumenyse, kaip šio parazito linijos hDELURB2 atstovai (**IV straipsnis**). Ši hipotezė ir kitos genetiškai artimos parazitų linijos (*Haemoproteus*

tartakovskyi Valkiūnas, 1986*, Haemoproteus lanii* Mello, 1936*, Haemoproteus balmorali* Peirce, 1984) gali būti testuojamos siekiant geriau suprasti filogenijos tinkamumą nuspėjant egzoeritrocitinį parazitų vystymąsi.

hEMCIR01 (*H. dumbbellus*) yra filogenetiškai artima linija hROBIN linijai (*H. attenuatus*) (**IV straipsnis**) ir abiejų rūšių parazitai formuoja merontus (**III, IV straipsniai**, (Iezhova, 1994)), bet ne megalomerontus. *Haemoproteus passeris* Kruse, 1890 taip pat formuoja merontus (Peirce, 1976) ir visos trys parazitų rūšys filogenetiškai yra artimos (**I–IV straipsniai**). Šios *Haemoproteus* parazitų rūšys galimai formuoja tik merontus, o dauguma *Haemoproteus* parazitų formuoja megalomerontus. *Haemoproteus fringillae*, taip pat genetiškai artimas minėtoms rūšims, tikėtina, kad formuoja merontus. Merontai, kurie buvo rasti *H. majoris* ir *Haemoproteus* spp. koinfekcijoje, spėjama, kad būdingi *H. fringillae* ar *Haemoproteus* spp., priklausomai nuo individo (**V straipsnis**). *H. majoris* parazitas formuoja tik megalomerontus (**I, V straipsniai**, Ilgūnas et al., 2019). Tai gali būti tam tikras merontų formavimo dėsningumas; tačiau reikia atlikti daugiau tyrimų su filogenetiškai artimais ir tolimais parazitais norint patikrinti šią hipotezę.

IŠVADOS

1. Megalomerontų morfologija ir vystymosi lokacija šeimininkuose skyrėsi trijų tritų parazitų rūšių (*H. majoris, H. pastoris* and *H. hirundinis*) atvejais, o dydis varijavo nuo 10 µm (labai jaunos stadijos) iki 800 µm.
2. *Haemoproteus attenuatus, H. dumbbellus* ir *Haemoproteus* spp. (parazitai neįdentifikuoti iki rūšies) merontai daugiausiai vystėsi paukščių plaučių kapiliaruose ir neviršiijo 100 µm.
3. Nei vienos tirtos *Haemoproteus* rūšies parazitai nevystė ir merontų, ir megalomerontų.
4. *Haemproteus* genties parazitai vysto merontus ir megalomerontus stuburinio šeimininko smegenyse (cerebralinė haemoproteozė).
5. Parazitemijos intensyvumas nėra susijęs su egzoeritrocitinių stadijų vystymu ir neturėtų būti laikomas patikimu parametru atrenkant paukščių individus egzoeritrocitinio *Haemoproteus* parazitų vystymosi tyrimams.
6. Chromogeninės *in situ* hibridizacijos metodas buvo būtinas norint patvirtinti matomų parazitų struktūrų gentį bei ieškant smulkių, netipinių formų *Haemoproteus* parazitų stadijų
7. Dalinės citochromo *b* geno sekos analizė neatskleidė egzeoeritrocitinio *Haemproteus* genties parazitų vystymosi dėsningumų, susijusių su parazitų lokacija šeimininko organuose. Tačiau tokia analizė rodo, kad yra grupė genetijų linijų, kurių parazitai formuoja tik merontus bei, kad

artimai susijusių genetinių linijų priklausančių tai pačiai parazitų rūšiai individai vysto panašios morfologijos egzoeritrocitines stadijas. Į tai verta atkreipti dėmesį taksonominių tyrimų metu ateityje.

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ADDITIONAL SCHORLARLY WORKS NOT ON THE SUBJECT OF THE DISSERTATION

Published articles not included in the dissertation.

The articles in journals with an impact factor and referred in the Clarivate Analytic Web of Science database:

1. Ciloglu, A., Ellis, V. A., **Duc, M.**, Downing, P. A., Inci, A., Bensch, S. 2020. Evolution of vector transmitted parasites by host switching revealed through sequencing of *Haemoproteus* parasite mitochondrial genomes. *Molecular phylogenetics and evolution*. 153, 106947. doi: 10.1016/j.ympev.2020.106947. (Q2 quartile; IF: 4.286).
2. Hellgren, O., Kelbskopf, V., Ellis, V. A., Ciloglu, A., **Duc, M.**, Huang, X., Lopes, R. J., Mata, V. A., Aghayan, S. A., Inci, A., Drovestski, S. V. 2021. Low MSP-1 haplotype diversity in the West Palearctic population of the avian malaria parasite *Plasmodium relictum*. *Malaria journal*. 20(1), 1-9. doi: 10.1186/s12936-021-03799-8. (Q2 quartile; IF: 3.222),
3. Valkiūnas, G., Ilgūnas, M., Bukauskaitė, D., **Duc, M.**, Iezhova, T. A. 2021. Description of *Haemoproteus asymmetricus* n. sp. (Haemoproteidae), with remarks on predictability of the DNA haplotype networks in haemosporidian parasite taxonomy research. *Acta Tropica*. 218, 1-16. doi: 10.1016/j.actatropica.2021.105905. (Q2 quartile; IF: 3.222).
4. Valkiūnas, G., **Duc, M.**, Iezhova, T. A. 2022. Increase of avian *Plasmodium circumflexum* prevalence, but not of other malaria parasites and related haemosporidians in northern Europe during the past 40 years. *Malaria journal*. 21(1), 1-11. doi: 10.1186/s12936-022-04116-7. (Q2 quartile; IF: 3.469).
5. Ellis, V. A., Kalbskopf, V., Ciloglu, A., **Duc, M.**, Huang, X., Inci, A., Bensch, S., Hellgren, O., Palinauskas, V. 2022. Genomic sequence capture of *Plasmodium relictum* in experimentally infected birds. *Parasites and vectors*. 15(1), 1-12. doi: 10.1186/s13071-022-05373-w. (Q1 quartile; IF: 4.047).
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7. Chagas, C. R. F., **Duc, M.**, Gutiérrez-Liberato, G. A., Valkiūnas, G. 2023. Host cells of *Leucocytozoon* (Haemosporida, Leucocytozoidae) gametocytes, with remarks on the phylogenetic importance of this

character. *Pathogens*. 12(5), 712. doi: 10.3390/pathogens12050712 (Q2 quartile; IF: 4.531).

International conference presentation not on the subject of the dissertation.

1. **Duc M.**, Treinys R., Bernotienė R., Kazak M., Chagas C. R. F., Bukauskaitė D. August 21-26, 2022. Identified vectors transmitting haemoproteid parasites of diurnal raptors. *15th International Congress of Parasitology (ICOPA)*. Copenhagen, Denmark. Poster presentation.

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Academics degrees

2019 Master's degree: Biology, option Animal Ecology, Lund University, Lund, Sweden.

2017 Bachelor's degree: Ecology, Biology of Organisms (Licence EBO, Ecologie, Biologie des Organismes), Montpellier University, Montpellier, France.

2016 Higher National Diploma: Environmental engineering (DUT Génie Biologique, option Génie de l'Environnement), Institute of Bretagne Occidentale University, Brest, France.

2015 Higher School Preparatory Class: BCPST (Biology, Chemistry, Physics, Earth's Sciences), Chateaubriand High School, Rennes, France.

Scholarships, awards, recognition

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2022 Scholarship for academic achievements from the Research Council of Lithuania. 2022, no. P-DAP-23-164.

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2023 01 08 – 2023 01 27 Musée National d'Histoire Naturelle (MNHN, Paris, France) to access the haemosporidians collection (Protists), under the EU SYNTHESYS+ grant.

2022 11 20 – 2022 12 10 Natural History Museum (NHM, London, United Kingdom) to access the haemosporidians collection (Protists), under the EU SYNTHESYS+ grant.

2021 10 12 – 2021 12 10 Institute of Pathology, University of Veterinary Medicine (Vienna, Austria) to learn and perform the chromogenic *in situ* hybridization, and further study histological procedures.

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Scientific publications

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Scientific conferences

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Research projects

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2021 – 2024 Lithuanian Research Council. Grant no. P-MIP-21-76. *Mechanisms of transmission of avian haemoproteosis*. Principal investigator Dr. Carolina R.F Chagas. Junior Researcher in the primary project implementer.

2020 – 2022 Lithuanian Research Council. Grant no. P-MIP-20-217. *Determination of vectors transmitting haemoproteid parasites of diurnal raptors.* Project principal investigator Dr. Bukauskaitė Dovilė. Junior Researcher.

2019 – 2022 European Research Council, Advanced Grant (HORIZON). Grant no. 742646. Immunity in ecology and evolution: *Hidden costs of disease, immune function, and their consequences for Darwinian fitness*. Project principal investigator Prof. Dennis Lennart Hasselquist. Subcontract implementor in Lithuania.

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PAPER I

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Patterns of *Haemoproteus majoris* (Haemosporida, Haemoproteidae) megalomeront development

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ABSTRACT

Blood parasites of the genus *Haemoproteus* (Haemosporida, Haemoproteidae) are cosmopolitan and prevalent in birds. Numerous species and lineages of these pathogens have been identified. Some of the infections are lethal in avian hosts mainly due to damage of organs by tissue stages, which remain insufficiently investigated. Several closely related lineages of *Haemoproteus majoris*, a common parasite of passeriform birds, have been identified. One recent study described megalomeronts of unique morphology in the lineages hPHYBOR04 and hPARUS1 of *H. majoris* and suggested that the similar tissues stages might also be features in other phylogenetically closely related lineages of the same parasite species. This study aimed to test if (i) megalomeronts are present during the development of the lineage hPHSIB1 of *H. majoris* and if (ii) they are similar to the other investigated lineages of this species in regard of their morphology and location in organs. One adult wood warbler *Phylloscopus sibilatrix*, an Afrotropical migrant, naturally infected with *H. majoris* lineage hPHSIB1 was wild-caught after seasonal spring migration and screened using microscopic examination of blood films and histological sections of organs as well as using PCR-based testing. Bayesian phylogenetic analysis placed the lineages hPHSIB1, hPHYBOR04 and hPARUS1 in one, well-supported clade. Parasitaemia was high (6.5%) in the examined wood warbler, numerous megalomeronts were found in kidneys, and a few in the intestine. Megalomeronts of the lineage hPHSIB1 were morphologically hardly distinguishable from those of lineages hPHYBOR04 and hPARUS1; only negligible differences in the maturation stage of the cytomeres were seen. The kidneys were the main location site of the megalomeronts in all three lineages of this parasite species. This study shows that closely related lineages of *H. majoris* produce megalomeronts of similar morphology and predominant location in kidneys, while the normal function of this organ may be affected by the presence of numerous large megalomeronts. Megalomeronts of different avian *Haemoproteus* species are markedly variable in morphology and location, but phylogenetically closely related lineages possess cryptic megalomeronts. This finding suggests that phylogenies based on partial *cytb* gene could provide information for prediction of patterns of exo-erythrocytic development of closely related *Haemoproteus* parasites and are worthy of attention in planning haemosporidian parasite tissue stage research.

1. Introduction

Avian haemosporidian parasites have traditionally been classified in four different families, including Haemoproteidae, containing one genus, *Haemoproteus* (Valkiūnas, 2005). Parasites of different subgenera of *Haemoproteus* are transmitted on all continents except for Antarctica (Clark et al., 2014). They are transmitted by biting midges of *Culicoides* (species of *Parahaemoproteus* subgenus) and louse flies of the Hippoboscidae (species of *Haemoproteus* subgenus) (Atkinson et al., 2008).

Recent molecular studies have pointed out the remarkable diversity of haemosporidian parasites and addressed numerous issues regarding their phylogenetic relationships, evolution and molecular identifications (Clark et al., 2014; Pacheco et al., 2018; Videvall, 2019). Early molecular studies often questioned the value of microscopic examination in haemosporidian research (Jarvi et al., 2002; Richard et al., 2002). However, currently the research community is in agreement that both molecular and microscopic approaches have strong and weak points in haemosporidian research (Valkiūnas et al., 2008) and ideally should be applied in parallel (Ciloglu et al., 2016; Clark et al., 2015). As currently used PCR-based methods normally cannot identify the differences between different developmental stages of haemosporidians, microscopic examination is essential when studying the full life cycle of these organisms (Bukauskaitė et al., 2019; Valkiūnas et al., 2009; Valkiūnas and Iezhova, 2017).

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0001-706X/

Former studies, which were based mainly on limited microscopic observations, often considered *Haemoproteus* parasites to be relatively benign in birds (Bennett et al., 1993). Recently, these pathogens have attracted the attention of researchers after well-documented molecular reports of lethal cases of haemoproteosis caused by different lineages of *Haemoproteus minutus* in captive parrots, a non-adapted avian host for this parasite (Ortiz-Catedral et al., 2019). It was shown that *H. minutus* produced large megalomeronts which inflicted massive damage to various internal organs, particularly the heart and gizzard. Massive haemorrhagic lesions were reported in dead individuals. In these well-documented cases, *H. minutus* could not complete its development and produce invasive merozoites resulting in the absence of parasitaemia (Ortiz-Catedral et al., 2019). However, little remains known about the exo-erythrocytic development and its patterns for the great majority of avian *Haemoproteus* parasites (Valkiūnas and Iezhova, 2017). Recent studies have shed some light on the exo-erythrocytic stages in some *Plasmodium* (Himmel et al., 2020; Ilgūnas et al., 2019b, 2019a, 2016; Palinauskas et al., 2016) and *Haemoproteus* parasites (Groff et al., 2019; Himmel et al., 2019; Ilgūnas et al., 2019), using histological examination of organs, in situ hybridization and laser microdissection of megalomeronts. The latter two methods were essential to prove generic and specific identification of reported tissue stages (Dinhopl et al., 2011; Himmel et al., 2019; Ilgūnas et al., 2019), which are of unusual morphology and were formerly considered as belonging to non-haemosporidian infections, for example *Besnoitia* species (Bennett et al., 1993) or *Leucocytozoon* spp. (Desser and Fallis, 1967).

Ilgūnas et al. (2019) discovered megalomeronts in two lineages of *Haemoproteus majoris*; hPHYBOR04 megalomeronts were only found in kidneys of a fieldfare *Turdus pilaris* (Turdidae), whereas hPARUS1 megalomeronts were found in several organs (kidneys, liver, lungs, spleen) of a great tit *Parus major* (Paridae). In both avian hosts, the parasites were morphologically similar, with the highest abundance of megalomeronts seen in kidneys. Based on data from the MalAvi database, several closely related lineages have been reported in *H. majoris,* which parasitize several different species of passeriform birds (Bensch et al., 2009); these lineages formed a robust clade in phylogenetic trees based on partial cytochrome *b* (*cytb*) sequences (Nilsson et al., 2016). A hypothesis was suggested that partial *cytb* gene phylogenies might indicate some patterns of exo-erythrocytic development (Ilgūnas et al., 2019). The investigation of a wood warbler (*Phylloscopus sibilatrix,* Phylloscopidae) revealed an infection of another common lineage of the species *H. majoris* – hPHSIB1. Wood warblers are long distance migrants that winter in tropical Africa and breed in Europe (Shirihai and Svensson, 2018). Through ring recoveries, isotope analyses and geolocalisation, the wintering grounds of this species have been determined in sub-Sahara regions, from Congo basin to Sierra Leone (Hobson et al., 2014; Tøttrup et al., 2018). *Haemoproteus* parasites have often been found in wood warblers in Europe (Bennett et al., 1982; Bishop and Bennett, 1992), and prevalence of this infection has been reported to be 19.2% ($n = 26$) in Lithuania (Valkiūnas, 1985). The lineage hPHSIB1 has been recorded in this bird species in other countries as well (Bensch et al., 2009, MalAvi database). We used this opportunity to histologically examine the organs of this wood warbler, aiming to determine if (i) megalomeronts are present during the development of the lineage hPHSIB1 of *H. majoris* and if (ii) they are similar to the other investigated lineages of this species in regard of their morphology and location in organs.

2. Material and methods

2.1. Study site and sample collection

The infected wood warbler was caught using mist nets in Labanoras Regional Park (55°12′25.77″ N, 25°56′26.47″ E), Švenčionys district, Lithuania on the 11th June 2019. This bird looked healthy during visual observation. A blood sample of approximately 50 µl was taken from the branchial vein, fixed in SET-buffer and stored at 4 °C in field and at −20 °C in the laboratory for further molecular analyses. Eight blood films were also prepared on slides, fixed by immersion in absolute methanol for one second and stained with Giemsa according to Valkiūnas (2005). The presence of parasite and species identification were achieved by microscopic examination of blood films in the field and later confirmed by PCR-based testing and sequencing in the laboratory (see description below). This individual bird was euthanized by decapitation according to permits (see Ethical statement) and its internal organs were collected for histological investigation.

2.2. Histological examination

The brain, heart, lungs, liver, kidneys, pectoral muscles, testis, stomach and intestine of the wood warbler were collected and fixed in 10% neutral formalin in the field. In the laboratory, the organs were processed using traditional histological procedures and then paraffin wax-embedded. The blocks were sectioned at 4 µm. The sections were mounted on glass slides, air-dried, deparaffinized and stained with haematoxylin-eosin (H&E) to be used for microscopic examination. Detail description of histological procedures were described by Lillie (1965) and Valkiūnas (2005).

2.3. Morphological and genetical parasite analysis

2.3.1. Blood stages

Blood films were screened using a BX41TF light microscope and images were taken using an Olympus DP12 digital camera and the Olympus DP-SOFT software (Olympus, Tokyo, Japan). The blood films were analysed for 15–20 min at medium magnification (x400) followed by examination of 100 microscope fields at high magnification (x1000). The number of parasites per 1000 red blood cells (in percentage) determined the intensity of parasitaemia (Godfrey et al., 1987). Voucher preparations of blood stages (accessions 49,228 NS – 49,235 NS) were deposited at the Nature Research Centre, Vilnius, Lithuania.

2.3.2. Exo-erythrocytic stages

The stained histological sections of all sampled organs were examined with a light microscope BX41TF equipped with an Olympus DP12 digital camera and the image software Olympus DP-SOFT (Olympus, Tokyo, Japan). Each histological section was screened entirely at low magnification (x200), then at medium magnification (x400) and finally at the highest magnification (x1000). Images of the tissue stages were prepared at different magnifications (x100, x200, x400 and x1000). The parasite measurements were taken at different magnification to determine the size of the megalomeront and the width of the capsule-like wall. Voucher preparations of tissue stages (accessions 49,236 NS – 49,241 NS) were deposited at the Nature Research Centre, Vilnius, Lithuania.

2.3.3. Deoxyribonucleic acid extraction, polymerase chain reaction and sequencing

An ammonium acetate protocol was used to extract total deoxyribonucleic acid (DNA) (Sambrook et al., 1989), which was diluted in TE buffer to a concentration of 25 ng/µl. The later was used as the template for the first amplification of the nested polymerase chain reaction (PCR) protocol. The standard primers (HaemNFI/HaemNR3 and HAEMF/HAEMR2), amplifying a fragment (479 bp) of the cytochrome *b* gene, temperature profiles and other details were as described in original protocol description (Bensch et al., 2000; Hellgren et al., 2004). A positive control (previously determined positive sample with a *Haemoproteus* sp. infection) and a negative control (ddH_2O) were used to control for possible contamination and false amplifications. 2 µl of the final PCR product were run on 2% agarose gel to check for positive amplifications. Those were then sequenced from both 3′ and 5′ ends with Big Dye Terminator V3.1 Cycle Sequencing Kit and ABI PRISM™

3100 capillary sequencing robot (Applied Biosystems, Foster City, California). Sequence chromatogram was checked in Geneious Prime 2020.0.5 software (https://www.geneious.com), where single infection is characterized by single peaks, while double peaks would attest of co-infections. The molecular result was compared to the result from blood film's microscopic examination; they were in agreement. The obtained DNA sequence (accession MT740752) was deposited in NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

2.4. Phylogenetic analysis

The sample sequence identification was done using the BLAST search in MalAvi database (Bensch et al., 2009). To build a phylogenetic tree and visualize the position of detected parasite lineage, sequences were retrieved from GenBank. In all, 45 lineages of morphologically identified *Haemoproteus* species, six of *Plasmodium* species and one of *Leucocytozoon* species (as outgroup) were used. The best fit model (GTR + G + I) was selected by the jModeltest-2.1.10 software (Darriba et al., 2012; Guindon and Gascuel, 2003) and run in Geneious with the MrBayes plugin v3.2.6 (Huelsenbeck and Ronquist, 2001). The analyses run for 5 million generations and sampled every 100th generation, while a 'burn-in' period representing the first 25% of trees was discarded for the construction of the consensus tree.

2.5. Ethical statement

Licenced researchers collected blood samples and organs under procedures approved by the Environmental Protection Agency, Vilnius, Lithuania (permit no. 23, 2019 04 19).

3. Results

3.1. Identification of the parasite and its phylogenetic relationships

Intensity of parasitaemia was 6.5%, with numerous gametocytes of various maturation observed in blood films (Fig. 1A-D). Presence of numerous young gametocytes (Fig. 1A) indicated the presence of exo-erythrocytic stages. Both microscopic examination (observation of *H. majoris* gametocytes, Fig. 1) and sequencing of the partial *cytb* gene (lineage hPHSIB1 of *H. majoris* was identified) suggested presence of a single haemosporidian infection. In phylogenetic analysis (Fig. 2), the reported lineage clustered with other *H. majoris* lineages (hPARUS1, hWW2, hPHYBOR04, hCCF5) and also the lineage hCWT4, which species identity remains unclear (Križanauskienė et al., 2006).

3.2. Exo-erythrocytic stages and pathology in organs

Only megalomeronts were seen, and they were present mainly in kidneys (Fig. 3). Megalomeronts were not found in the other organs, except the intestine (Fig. 4). Numerous megalomeronts at different stages of maturation were seen in the renal tissues of kidneys (Fig. 3). These parasites were big roundish bodies, which largest diameter reached up to 300 μm. Each megalomeront was covered with a prominent capsule-like wall (Figs. 3B-D, F, 4A-D), width of which varied between 1.8 and 11 μm in different sections. Numerous variously shaped cytomeres develop during the growth of the parasite (Fig. 3B, C). During megalomeront maturation and cytomere development, prominent non-stained spaces, which look like empty areas (Fig. 3A-B, E-F), occurred between cytomeres and the wall of megalomeronts. Numerous small budding merozoites measuring approximately 2 μm in diameter were present as megalomeronts reached maturity (Fig. 3G, H). Mature megalomeronts were tightly packed with numerous unicellular merozoites (Fig. 3H).

In intestine, the megalomeront was located in the muscular part on the outside wall of the organ (Fig. 4A, B). It was surrounded by a capsule-like wall (Fig. 4A-D), but was of a smaller size than those seen in the kidneys (diameter was 88 μm) and was of oval shape, probably due to pressure of musculature. A striated layer was visible surrounding the hyaline capsule-like wall of the megalomeront (Fig. 4C, D). No megalomeronts or any other exo-erythrocytic meronts were found in the other examined organs.

Liver and spleen of infected bird were of slight black colour and enlarged due to presence of pigment originating from gametocytes. Pathological changes of other organs were not observed visually during the dissection. There was no evidence of tissue necrosis. Inflammatory cell infiltration was not seen during microscopic examination of organs. Intensive infection and damage by large-size megalomeronts in kidneys seem to be the main pathological danger during this infection.

4. Discussion

The key result of this study is the discovery of megalomeronts of the lineage hPHSIB1 of *H. majoris*. It was shown that the megalomeronts preferably developed in the kidneys of the wood warbler (family Phylloscopidae), and were morphologically similar to megalomeronts described in lineages hPHYBOR04 (in a fieldfare, Turdidae) and hPARUS1 (in a great tit, Paridae), both of which also preferably develop in the kidneys (Ilgūnas et al., 2019). As parasites of these three lineages appeared in the same clade during phylogenetic analysis (Fig. 2), these findings suggest that (i) the closely related lineages of the same species likely have similar patterns of exo-erythrocytic development (in regard of morphology and location in organs) in different avian hosts and (ii) the well-supported clades in phylogenies based on partial *cytb* gene sequences might indicate features of exo-erythrocytic stages in non-investigated, closely related lineages.

Research of exo-erythrocytic stages of haemosporidian parasites is challenging. It requires application of histological procedures, which are time-consuming, and also euthanasia of birds (Atkinson et al., 2008;

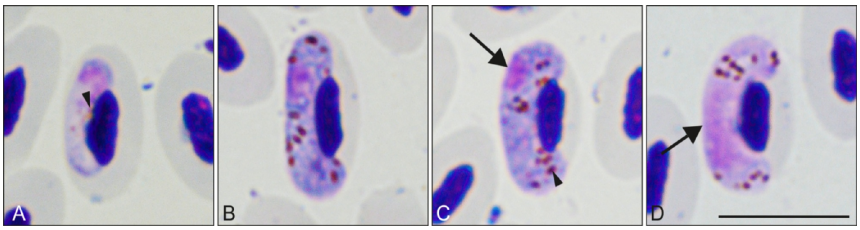

Fig. 1. Gametocytes of *Haemoproteus majoris* (lineage hPHSIB1) from naturally infected wood warbler *Phylloscopus sibilatrix*: A – young gametocyte, B – advanced growing macrogametocyte, C – mature macrogametocyte, D – mature microgametocyte. Note that mature gametocytes are halteridial in shape, they fill poles of infected erythrocytes completely and slightly displace host cell nuclei laterally. Triangle arrows – nuclei of parasites. Triangle arrowheads – pigment granules. Giemsa-stained thin blood films. Scale bar = 10 μm.

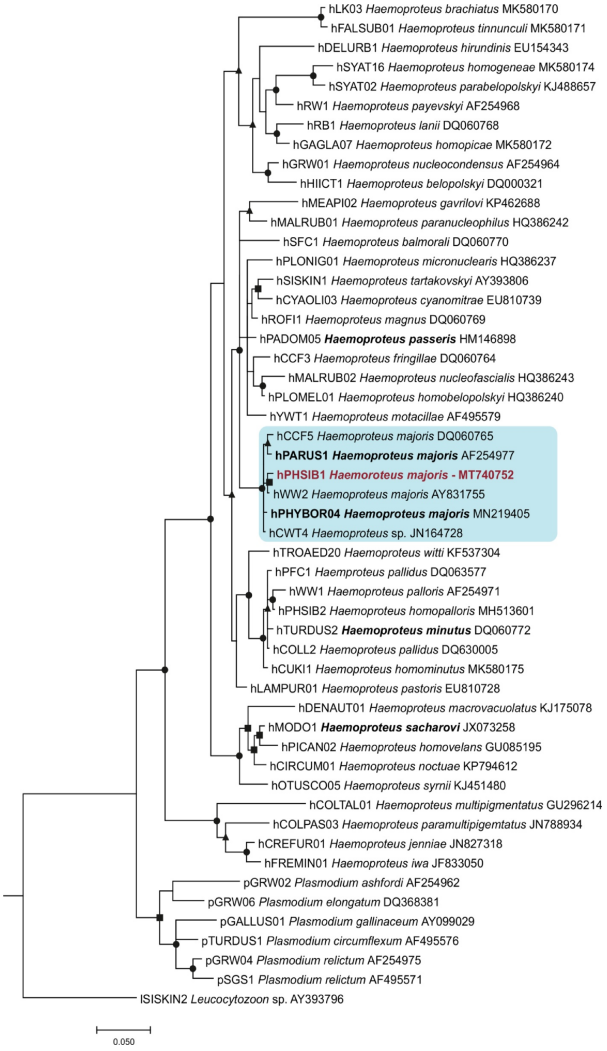

Fig. 2. Bayesian phylogenetic tree constructed using partial cytochrome *b* sequences of 45 lineages of *Haemoproteus*, six lineages of *Plasmodium*, with one *Leucocytozoon* sp. lineage used as outgroup. Posterior probabilities higher than 0.6, 0.7 and 0.9 were indicated with triangle, square and circle symbols, respectively. The blue box indicates closely related parasite lineages of the *Haemoproteus majoris* group. Bold font indicates names of *Haemoproteus* species for which megalomerонts were formerly reported; note that these parasites are present in different clades and have markedly different megalomeronts morphology (see Discussion for further explanation). Lineages names were provided (according to MalAvi database), followed by parasite species names and sequence GenBank accession number. The parasite lineage studied in this paper is given in red font. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Valkiūnas and Iezhova, 2017). On the contrary, parasite DNA sequence information is easier to access through bird blood samples, and is already known for many haemosporidian parasite species (Bensch et al., 2009). This sequence information can be used for predicting possible patterns of exo-erythrocytic development in lineages, which remain non-investigated in this regard. Available data show that *H. majoris* (Ilgūnas et al., 2019, this study), *H. minutus* (Himmel et al., 2019), *H. passeris* (Paperna and Gill, 2003) and *H. sacharovi* (Farmer, 1964) produce megalomeronts of markedly different morphology and location (Valkiūnas and Iezhova, 2017), and these parasite sequences appear in different distinct clades (Fig. 2). Based on these data we suggest that other *Haemoproteus* spp. lineages, which are closely related to the mentioned parasites, also might have similarly developing megalomeronts in regards of morphology and site of their development. In other words, the phylogenetic analysis would be of predictive value in regard of the megalomeront development. Furthermore, phylogenies based on partial *cytb* gene distinguish *Haemoproteus* parasites groups with similar patterns of ookinete morphological transformation and provide opportunities to predict rate of ookinete maturation without direct research of sporogony (Chagas et al., 2019). Available limited

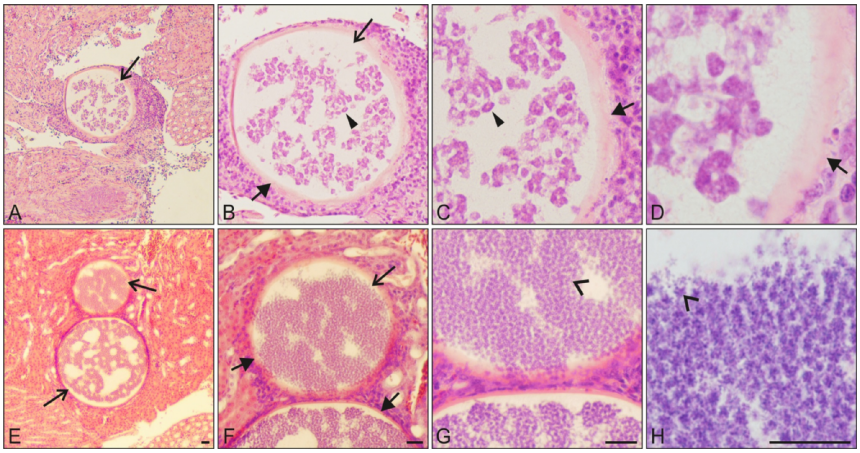

Fig. 3. Megalomeronts of *Haemoproteus majoris* (lineage hPHSIB1) in kidneys of a naturally infected wood warbler *Phylloscopus sibilatrix*: A-D – a growing megalomeront with early cytomeres (note that nuclei are large, which is an indication of parasite growth and intensive chromatin multiplication); E-H – two nearly mature megalomeronts with already less evident cytomeres and chromatin arranging in distinct developing merozoites (G, H). Note the prominent capsular-like wall (B-D, F) and the numerous variously shaped cytomeres (B-C). Parasites were shown at four different magnifications: A, E x100; B, F x200; C, G x400 and D, H x1000. Simple arrows – megalomeront. Triangle arrows – capsule-like wall. Triangle arrowheads – cytomeres. Simple arrowheads – aggregations of chromatin on final stage of merozoite maturation (budding merozoites). All scale bars = 20 μm.

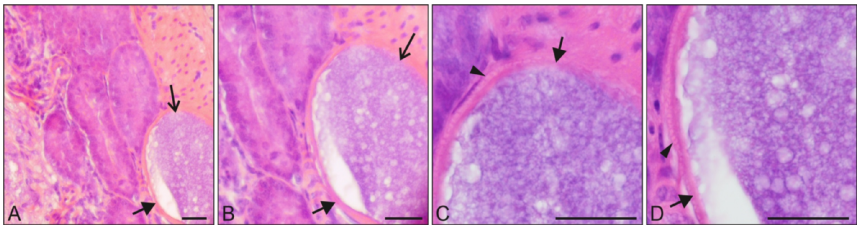

Fig. 4. Young megalomeront of *Haemoproteus majoris* (lineage hPHSIB1) in intestine of a naturally infected wood warbler *Phylloscopus sibilatrix*. Note that cytomeres only started to appear and the nuclear material division is not well-visible, which is an indication of early stage of development of the parasite. Capsular-like wall is well seen, and a striated area, which is likely of host origin, adheres to the wall. The same parasite was shown at different magnifications: A x200, B x400 and C, D x1000. Simple arrow – megalomeront. Triangle arrows – capsule-like wall. Triangle arrowheads – striated outer area. All scale bars = 20 μm.

data about avian haemoproteids (Fig. 2; Himmel et al., 2019; Ilgūnas et al., 2019; Valkiūnas and Iezhova, 2017) indicate that the patterns of exo-erythrocytic development and megalomeront formation might be of phylogenetic value and thus is worth more attention in haemosporidian parasite research. Further studies and gathering of information on the exo-erythrocytic development of other haemoproteids is needed for better understanding if and how application of DNA sequence information can be used to predict possible patterns of exo-erythrocytic development in haemosporidians. This is important to speed up and simplify the research on haemosporidian tissue stages, particularly in wildlife.

Few megalomeronts were observed in the intestine in this study (Fig. 4), while kidneys were the main site for development of megalomeronts in both studies of *H. majoris* lineages (Ilgūnas et al., 2019; this study). In all studied lineages of *H. majoris*, megalomeronts in kidneys were bigger in size than those found in other organs, probably indicating an optimal environment for their development. Thick capsular-like wall also develops in megalomeronts of *H. minutus* (Ortiz-Catedral et al., 2019) and *Leucocytozoon* species (Valkiūnas and Iezhova, 2017). It was also proved to be of host origin in *H. minutus* (Himmel et al., 2019), which probably is the case in other haemoproteid infections as well. This might explain the absence of host response and inflammation around intact developing megalomeronts before their rupture (Atkinson et al., 1988). Such wall was previously seen and referred to as a hyaline wall in many species of the Haemoproteidae and Leucocytozoidae, but it is absent in species of Plasmodiidae and Garniidae (Atkinson et al., 2008; Valkiūnas and Iezhova, 2017).

It is interesting to note that a striated layer surrounding the more hyaline capsule of the megalomeronts was visible in the intestine (Fig. 4C, D), but not in the kidneys (Fig. 3). As only a single *Haemoproteus* infection was detected in this bird individual both by microscopic examination and molecular method, it would be difficult to speculate about the possible presence of other parasites in our sample; it is likely that all megalomeronts reported in this study belonged to the same parasite. It is important to note that recent molecular studies have proved that hyaline wall in megalomeronts of *Haemoproteus* parasites is

of host origin (Himmel et al., 2019; Ilgūnas et al., 2019). As the striated area is located outside of the hyaline wall of the megalomeronts, it is certainly of host origin as well. Development of this striated area probably depends on the parasite location in intestine. Further electron microscopy investigations are needed to make this issue clearer.

Few studies have found exo-erythrocytic stages of different *Haemoproteus* species, and these were mainly seen in non-passerine birds (Valkiūnas and Iezhova, 2017). The megalomeronts of all investigated lineages of *H. majoris* in different avian hosts were morphologically similar (Ilgūnas et al., 2019; Figs. 3–4), but they were markedly different from those in other haemoproteid species mainly due to the markedly irregular shape of developing cytomeres, and the occurrence of large poorly stained areas, which look like empty spaces, separating the cytomeres from each other and off the capsular-like wall (Fig. 3B-D, F). These spaces certainly are not artefacts as they regularly contain tiny shreds inside, indicating parasite structures. Furthermore, the same structure of megalomeronts was described in other lineages of *H. majoris* (Ilgūnas et al., 2019). Interestingly, megalomeronts of *H. mansoni* (synonym of *H. meleagridis*), *H. handai* (synonym of *Parahaemoproteus desseri*), *H. halcyonis* and *H. lophortyx* were seen frequently in the skeletal muscle tissues in naturally infected birds and during experimental infections (Atkinson et al., 1988, 1986; Cardona et al., 2002; Miltgen et al., 1981; Peirce et al., 2004), but this was not the case with *H. majoris*. This indicates that the location of megalomeronts might be characteristic to parasite species as megalomeronts of the three investigated lineages of *H. majoris* were not seen in muscles of any avian hosts belonging to different families.

Finally, it is worth mentioning the presence of the budding merozoites, which were visible at the final stage of the maturation of megalomeronts (Fig. 3G, H). Similar structures (described as 'tiny specks of chromatin') were observed in maturing *H. mansoni* megalomeronts, which were located in the skeletal muscle of domestic turkeys at 17 day post experimental infection with sporozoites (Atkinson et al., 1986). This suggests that some features of megalomeront maturation and merozoite formation are similar amongst different species of *Haemoproteus*, however it remains insufficiently investigated.

5. Conclusion

This study and analysis of available published information show that the three closely related lineages of *H. majoris* (hPHSIB1, hPHYBOR04 and hPARUS1) produce megalomeronts of similar morphology and preferable location of development (kidneys) in three species of avian hosts belonging to the families Phylloscopidae, Turdidae and Paridae, respectively. These parasites were cryptic in regard to the megalomeront stage in different passeriform bird species, indicating that the megalomeront morphology and preferable location in kidneys likely are regulated by the parasite characteristics rather than these being host-controlled features. This study suggests that phylogenies based on partial *cytb* gene distinguish groups of closely related parasite lineages, which have similar patterns of exo-erythrocytic development, providing opportunities to use the sequence information to predict possible patterns of exo-erythrocytic development.

CRediT authorship contribution statement

Mélanie Duc: Data curation, Formal analysis, Investigation, Writing - original draft. **Mikas Ilgūnas:** Investigation, Validation, Methodology, Writing - review & editing. **Gediminas Valkiūnas:** Conceptualization, Methodology, Funding acquisition, Validation, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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PAPER II

First report of *Haemoproteus* (Haemosporida, Haemoproteidae) megalomeronts in the brain of an avian host, with description of megalomerogony of *Haemoproteus pastoris*, the blood parasite of the common starling.

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First Report of *Haemoproteus* (Haemosporida, Haemoproteidae) Megalomeronts in the Brain of an Avian Host, with Description of Megalomerogony of *Haemoproteus Pastoris*, the Blood Parasite of the Common Starling

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Simple Summary: Birds are hosts to diverse blood parasites belonging to many taxonomic groups. Among them, numerous haemosporidian parasites of the genus *Haemoproteus* are transmitted globally. These pathogens develop in the blood and internal organs of birds. The blood stages (gametocytes) are known for about 150 described species, but the tissues stages or exo-erythrocytic stages (meronts and megalomeronts) are known only fragmentarily for about 10% of the described species. Knowledge on merogony is important in avian medicine for better understanding of pathologies during haemoproteosis. This study reported and characterized the megalomeronts of *Haemoproteus pastoris*, a parasite of the widespread Common starling (*Sturnus vulgaris)*. Parasites were identified using molecular and microscopy examination tools. Five individual naturally infected birds were sampled, and their organs were examined histologically. Megalomeronts were found in eight different organs. The parasites were described and illustrated. The largest megalomeront, of all observed forms and shapes, reached 800 μm in length. Importantly, *Haemoproteus* megalomeronts were reported in the brain of avian hosts for the first time, indicating non-described pathology during avian haemoproteosis. This study contributes to a better understanding of the life cycle of avian haemoproteids and opens new perspectives in pathology research during avian haemoproteosis, which is important for birds' health.

Abstract: Species of *Haemoproteus* (Haemoproteidae, Haemosporida) are common bird pathogens. Recent molecular studies combined with histopathology research have reported development of megalomeronts of these parasites in various organs, sometimes resulting in the death of the avian host. Five Common starlings *(Sturnus vulgaris)* were found naturally infected with *Haemoproteus pastoris* lineage hLAMPUR01. The parasite was identified using microscopic examination of blood films and DNA sequences. Infected bird organs were investigated histologically for (i) the presence of exo-erythrocytic stages and (ii) the patterns of development (morphology and localization) in different host individuals. For the first time, megalomeronts of *Haemoproteus* parasites were seen developing in the brain, while numerous others at different stages of maturation were found in the intestine, pancreas, kidneys, lungs, esophagus, spleen, gizzard, and trachea. Megalomeronts were predominantly roundish or oval, up to 800 μm, they were surrounded by a capsular-like wall and developed asynchronously in the same bird individual. After megalomeront maturation and rupture, a massive infiltration of blood cells occurred, indicating the hemorrhagic processes. Review of available data showed that different *Haemoproteus* species produce markedly different megalomeronts, morphology of which can probably be predicted using phylogenetic analysis based on partial sequences of cytochrome *b* gene.

Keywords: haemosporidian parasites; birds; *Haemoproteus pastoris*; exo-erythrocytic development; megalomeronts; brain

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1. Introduction

Avian haemosporidians (Haemosporida, Apicomplexa) are endoparasites, which are transmitted by species of Diptera and develop in the blood cells and internal organs of vertebrates. Gametocytes of haemosporidians infect various blood cells, and their tissue stages or exo-erythrocytic stages (meronts and megalomeronts) develop in internal organs. Meronts are thin-wall structures about 50 µm in maximum length, while megalomeronts are big, usually roundish or oval structures often reaching more than 300 µm in diameter, surrounded by a thick-wall [1,2]. Haemosporidians are classified in four families: Plasmodiidae, Haemoproteidae, Garniidae, and Leucocytozoidae [1,2]. In terms of species and lineage diversity, *Haemoproteus* parasites of the Haemoproteidae outnumber the parasites found in other families (MalAvi database http://130.235.244.92/Malavi/ accessed on July 2021 [2,3]). Haemoproteids are cosmopolitan and parasitize birds of the majority of orders, on all continents except Antarctica (MalAvi database [2–5]).

The Common starling *(Sturnus vulgaris)* is a common bird species found in Europe, where it is native and widespread. This species is markedly invasive and has been introduced in Asia, Australia, Northern America, and Northern and South Africa [6]. Being an omnivore bird species, it can be considered a pest due to flocking in gardens and agricultural lands in some countries [6]. Diversity of haemosporidian parasites is low in the Common starling, with four lineages of *Plasmodium* and only one species of *Haemoproteus*, *Haemoproteus pastoris* lineage hLAMPUR01, described in this naturally infected host (MalAvi database [3]). Available information about this pathogen covers only molecular data (partial cytochrome *b* gene sequence) [3], morphology of blood stages (gametocytes) [1], and sporogony in *Culicoides* biting midges [7,8]. Exo-erythrocytic development of *H. pastoris* remains unknown.

Indeed, research on exo-erythrocytic stages of *Haemoproteus* parasites is scarce, starting in 1908 with the discovery of exo-erythrocytic stages of *Haemoproteus columbae* in pigeons [9], followed by a few other reports of tissue stages in several different parasite species [10–13]. Due to the predominant opinion about harmlessness of avian *Haemoproteus* infections [14], the research on exo-erythrocytic stages of *Haemoproteus* spp. remained slow for over a century [15–19]. However, interest of researchers regarding this issue was renewed by reports providing molecular evidence about avian *Haemoproteus* infections, which are responsible for mortality in birds due to damage caused by tissue stages [20]. Several recent molecular studies supplemented with histopathology research have proved that *Haemoproteus* parasites influence the health of the infected bird [20–24], but the mechanisms of pathologies might be different in different pathogen species and remain poorly understood.

Several previous studies suspected occurrences of large megalomeront-like structures in birds during *Haemoproteus* infections [1,13], but there was no proof that they belonged to *Haemoproteus*. It was believed that the structures might have been stages of other apicomplexan parasites, for example, *Besnoitia* spp. [14]. Recently, molecular techniques have been applied in parallel with histology research in order to prove that megalomeronts certainly develop in host tissues during haemoproteosis, indicating that this developmental stage is an important part in the life cycle of *Haemoproteus* parasites [18,25,26]. However, patterns of megalomerogony remain unclear during haemoproteosis. According to the available information, tissue stages of *Haemoproteus* spp. have been found in the gizzard, heart, intestine, kidneys, liver, lungs, proventriculus, skeletal muscle, and spleen [13,22]. To date, they have not been found in the brain, nor the reproductive organs, but these organs can be parasitized by other haemosporidians. For example, phanerozoites of *Plasmodium* sp. develop in the brain and megalomeronts of *Leucocytozoon* sp. parasitize the brain and reproductive organs of birds [1,13]. Moreover, the few investigated species of *Haemoproteus* have shown a remarkable morphological diversity of megalomeronts, with cytomeres being separated by septa (*H. passeris* [13]) or not (*H. majoris* [22,26]) and megalomeronts developing in tight clusters (*H. minutus* [20], *H. asymmetricus* [25]) or not (*H. syrnii, H. velans* [21,23]). Several recent studies published detail colorful illustrations of

developing and mature megalomeronts, including different stages of cytomeres formation and maturation of merozoites [22,24,25], while old publications have reported completely mature and ruptured megalomeronts for speculated *Haemoproteus* spp. [9–11,27]. Available limited information provides opportunities to start detailed comparative research on megalomerogony during avian haemoproteosis.

Further accumulation of data about exo-erythrocytic development of haemoproteids is needed. During this study, we sampled five Common starlings naturally infected with *Haemoproteus pastoris*, identified this parasite using microscopic examination and DNA barcoding sequences, and conducted histological examinations. We aimed to (i) determine the presence of exo-erythrocytic stages in this bird species during infection of one parasite lineage and (ii) gain information about the patterns of development (morphology and localization) in different host individuals. We also discussed available morphological information about megalomeronts of other *Haemoproteus* species in regard of their phylogenetic relationships.

2. Materials and Methods

2.1. Study Site and Samples

Common starlings were caught using permanent traps (large 'Rybachy' type, zigzag and funnel traps) and mist nets at the Ventes Ragas Ornithological station (55°20'38.93" N, 21°11'34.05" E), Lithuania in May 2019. This period corresponded to the beginning of the breeding season of Common starlings at the study site. Blood samples were collected by puncturing the branchial vein and fixed in SET-buffer [28] for further molecular analyses. A drop of fresh blood was used to prepare 2–13 blood films on ready-to-use glass slides. The films were fixed by immersion of the slides in absolute methanol for one second and then stained with 10% Giemsa [1]. In all, 19 Common starlings were sampled. Microscopic examination of blood films determined the presence of haemosporidians, and species of *H. pastoris* was identified [1]. Later, the species identification was confirmed by using molecular barcoding in the laboratory (see description below). Microscopic examination of blood films was performed using a BX61 light microscope (Olympus, Tokyo, Japan). Five microscopy-positive birds with gametocyte parasitemia between 1 and 26% of infected red blood cells (calculated according to [29]) were euthanized (by decapitation) and their organs were processed for histological examination.

2.2. Histological Examination

The brain, heart, intestine, pancreas, kidneys, liver, lungs, esophagus, pectoral muscles, spleen, gizzard, reproductive organs, and trachea were collected and fixed in 10% neutral formalin in the field. At the laboratory, the organs were processed using traditional histology techniques: dehydrating the samples in 96% EtOH, followed by a clarification in Isopropanol and embedded in paraffin blocks. For each organ, sections of 4 µm were prepared with a microtome, mounted on glass slides, air-dried, stained with hematoxylin–eosin (H&E) and covered with coverslip. Detailed histological procedures were described in [1,30].

All microscopic examinations of histological sections were performed in the laboratory using a light microscope BX41TF equipped with an Olympus DP12 digital camera and the image software Olympus DP-SOFT (Olympus, Tokyo, Japan). Each histological section was screened entirely at low (×200) and at medium magnification (×400). Depending on the organ size, about 0.5–1 mm depth of histological preparations was cut and examined. The highest magnification (×1000) was used to observe the structure of the megalomeronts. Different magnifications (×100, ×200, ×400, and ×1000) were used to prepare photographs aiming to better illustrate megalomeronts. The parasite measurements [diameter (for roundish-shape) and length x width (for oval-shape) of the megalomeronts, and thickness of capsular-like walll] were taken at most fitted magnifications, ×100, ×200,

or ×1000 depending on the size of the object. Voucher preparations of parasite gametocytes (accessions 49,288 NS–49,319 NS) and megalomeronts (accessions 49,320 NS–49,360 NS) were deposited at the Nature Research Centre, Vilnius, Lithuania.

2.3. DNA Extraction, PCR and Sequencing

DNA (deoxyribonucleic acid) was extracted using an ammonium acetate protocol [31], and diluted in 1X TE buffer to work at a concentration of 25 ng/μL. A nested PCR (polymerase chain reaction) was used to determine the lineage of the parasite by amplifying 478 bp of the cytochrome *b* gene (cyt *b*), using the standard primers HaemNFI/HaemNR3 and HaemF/HaemR2 for *Haemoproteus* and *Plasmodium* parasites. The parameters of the nested PCR were as described in original protocols descriptions [28,32]. For the first amplification, the extracted DNA was used as a template. To control for possible contamination and false amplifications, a positive control (a *Haemoproteus* sp. positive sample), and a negative control (ddH_2O) were used. Together with a dye, 2 μL of the final PCR product was run on a 2% agarose gel to check for positive amplification. Those were then sequenced from the 3′ end with a Big Dye Terminator V3.1 Cycle Sequencing Kit and ABI PRISM™ 3100 capillary sequencing robot (Applied Biosystems, Foster City, CA, USA). Sequences were checked in Geneious Prime 2020.0.5 (https://www.geneious.com, accessed on November 2020) for quality, identification of possible mixed infections (one peak for single infection, two or more peaks for mixed infections), and identification of lineage. The molecular results and blood film microscopy results were compared for species identification.

2.4. Phylogenetic Analysis

BLAST searches in MalAvi [3] and the NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/, accessed on November 2020) database were used for lineage identification using the obtained sequences. The following lineages sequences were retrieved from the GenBank database and used to build the phylogenetic tree: 30 lineages of *Haemoproteus* species, 6 of *Plasmodium* species, and 1 of *Leucocytozoon* species (lSISKIN2, as outgroup). Lineages of morphologically characterized parasites as well as *Haemoproteus* spp. with described exo-erythrocytic development were incorporated into the analysis for comparative purposes. The software jModeltest-2.1.10 [33,34] was used to select the best-fit model (GTR+G+I), which was run in Geneious with the MrBayes plugin v3.2.6 [35] for 5 million generations, and sampled every 100th generation, while discarding the first 25% of trees as a 'burn-in' period for the construction of the consensus tree.

3. Results

3.1. Parasite Identification and Phylogenetic Relationships

Of the 19 examined Common starlings, seven (prevalence of 36.8%) were *Haemoproteus* sp.-positive by microscopic examination of blood films. Other blood parasites were not seen, except for *Lankesterella* sp. in individual no. 3 (Table 1). All reported *Haemoproteus* infections were *H. pastoris* based on gametocyte morphology (Figure 1). Five intensively infected birds, with parasitemia ranging between 1 and 26% (Table 1), were used for investigation of tissue stages. Gametocytes on various stages of development (young Figure 1A, growing Figure 1 B,D and mature Figure 1 C,E) were seen in these birds, indicating asynchronous gametocytogony.

Table 1. Intensity of parasitemia and megalomeront-positive organs seen in the five dissected naturally infected Common starlings (*Sturnus vulgaris*). The number of visualized megalomeronts in histological sections (in parenthesis) and the stage of their development are shown. Note that reports of megalomeronts did not seem to be related to the intensity of parasitemia, as megalomeronts were not seen in individual no. 4, in which parasitemia was high (10%), but were present in bird no. 1, in which parasitemia was lower (1%). No megalomeronts were found in any of the individuals in the heart, liver, pectoral muscles, or the reproductive organs.

Individual Number	Parasitemia (%)	Brain	Intestine	Pancreas	Kidneys	Lungs	Esophagus	Spleen	Gizzard	Trachea
1	1	0 [a]	growing (2)	0	growing (1)	0	ruptured (1)	growing (1) ruptured (3)	0	0
2	2	0	growing (1)	0	0	0	0	growing (1)	0	growing (1)
3	3	0	growing (4) ruptured (4)	growing (1) mature (1) ruptured (1)	0	growing (1)	0	0	growing (1)	0
4	10	0	0	0	0	0	0	0	0	0
5	26	mature (1)	ruptured (2)	0	0	0	growing (1) ruptured (1)	0	growing (2)	0

[a] Organ examined, but megalomeronts were not found.

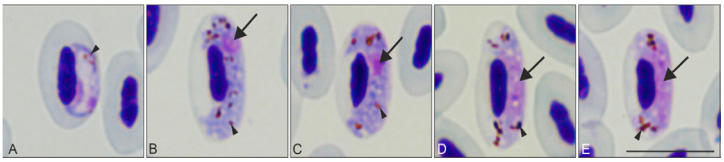

Figure 1. Gametocytes of *Haemoproteus pastoris* from naturally infected Common starlings *Sturnus vulgaris*. Young (**A**), growing (**B**,**D**), and mature (**C**,**E**) macrogametocytes (**B**,**C**) and microgametocytes (**D**,**E**) are shown. Note the presence of prominent pigment granules in gametocytes. The nuclei were compact and of sub-terminal position in macrogametocytes (**B**,**C**), but were diffuse and located centrally in microgametocytes (**D**,**E**). Ameboid extremities were visible in the growing gametocytes (**B**,**D**), and slight lateral displacement of the erythrocyte nuclei was seen in cells containing mature gametocytes (**C**,**E**). Triangle arrow (➧)—parasite nucleus. Triangle arrowhead (►)—pigment granules. Images were taken using Giemsa-stained blood films at ×1000 magnification. Scale bar = 10 μm.

Only the sequence of hLAMPUR01 lineage was identified in all dissected Common starlings for *Haemoproteus* parasites. This sequence clustered with the other sequences of haemoproteids belonging to subgenus *Parahaemoproteus* but was phylogenetically apart in relation to other *Parahaemoproteus* and *Haemoproteus* species for which megalomeronts are known (Figure 2). The sequence of pGRW11 of *Plasmodium relictum* was detected in individual 5.

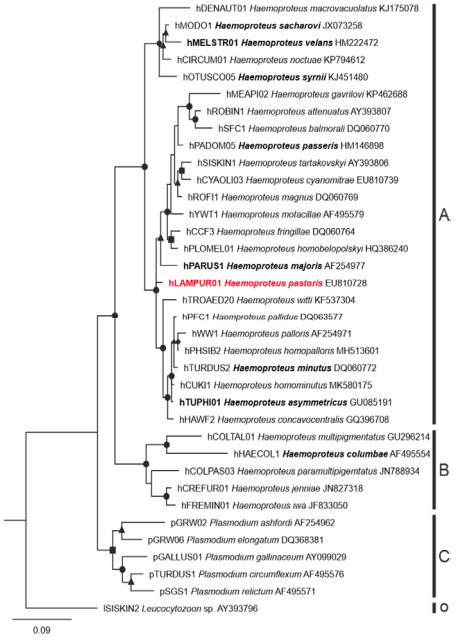

Figure 2. Bayesian phylogenetic tree of partial cytochrome *b* sequences of 30 lineages of *Haemoproteus* parasites, 6 lineages of *Plasmodium* spp., and 1 *Leucocytozoon* sp. lineage used as an outgroup. Vertical bars (A-O) represent parasite species of the subgenera *Parahaemoproteus* (**A**), *Haemoproteus* (**B**), parasites of the genus *Plasmodium* (**C**), and the outgroup, a *Leucocytozoon* sp. parasite (O). Bold font indicates names of *Haemoproteus* species and their corresponding lineage for which megalomeronts were previously reported; note that these parasites are present throughout the phylogeny and have markedly different megalomeront morphology (see Discussion section for further explanation). The parasite lineage studied in this work is given in red font. Posterior probabilities are provided with symbols: triangles—0.7–0.8; squares—0.8–0.9; rounds—0.9–1. Lineage names are given according to MalAvi database (http://130.235.244.92/Malavi/, accessed on July 2021), followed by their parasite species names and sequence GenBank accession numbers.

3.2. Description of Megalomeronts

No meronts were seen in the histological sections of the five individuals, while megalomeronts were found in four of the five examined individuals (Table 1). Each megalomeront was surrounded by a prominent capsular-like wall, the thickness of which varied depending on the size of the parasites (Figures 3–5). In total, combining all megalomeronts found per organs in all investigated starlings, the parasites were seen in the brain (1 parasite found in the cerebellum, Figure 3A–D), kidneys (1), lungs (1), intestine (13 in the mucosa and the muscularis, Figures 4E–H,M–P, 5D–F), pancreas (3, Figures 4I–L, 5A-C), esophagus (3 in the epithelium of the mucosa and the muscularis, Figure 1E–P), spleen (5, Figure 5G–I), gizzard (3 in the mucosa and the muscularis, Figure 4A–D), and the trachea (1). Megalomeronts were of similar morphology in all organs, however they were not seen in the same organs in each infected bird individual (Table 1). It is important to note that each megalomeront was considered as one entity, so if different sections of the same megalomeront were visualized, the parasite was still counted as the same one. For example,

images in Figure 3E–L represent one megalomeront from two different cuts, and it was thus counted as one for the count in Table 1. As such, it is difficult to assess in which part of the megalomeront the section was performed if one preparation was available. In other words, it is difficult to know if it was cut more in its center or closer to the periphery, except when consecutive sections were performed as in Figure 3E–L. This is why, mainly, the biggest diameter of megalomeronts can be estimated as a factual parasite size feature. As megalomeronts seemed to be most often of a roundish shape, the observed smaller parasites on flat histological cuts may have been growing ones, but also sections of the parasite made closer to its periphery.

The majority of megalomeronts were of a roundish shape when growing (Figure 3A–L), with reported minimum size of 120 µm and a maximum size of 520 µm in diameter. Oval-shape megalomeronts were also observed among growing parasites, but such forms were only seen in the gizzard and the intestine probably due to the orientation of the muscle fibers in the latter organs (Figure 4A–H). The maximum length and width were 380 and 167 µm, respectively, for the oval megalomeronts. In growing megalomeronts, developing cytomeres were seen located unevenly inside the megalomeront, with numerous cytomeres present in one part of the parasite and fewer in other parts of the same parasite (Figures 3E–L, 4A–H). The capsular-like wall was prominent, measuring between 2 and 12 µm in width at different sections.

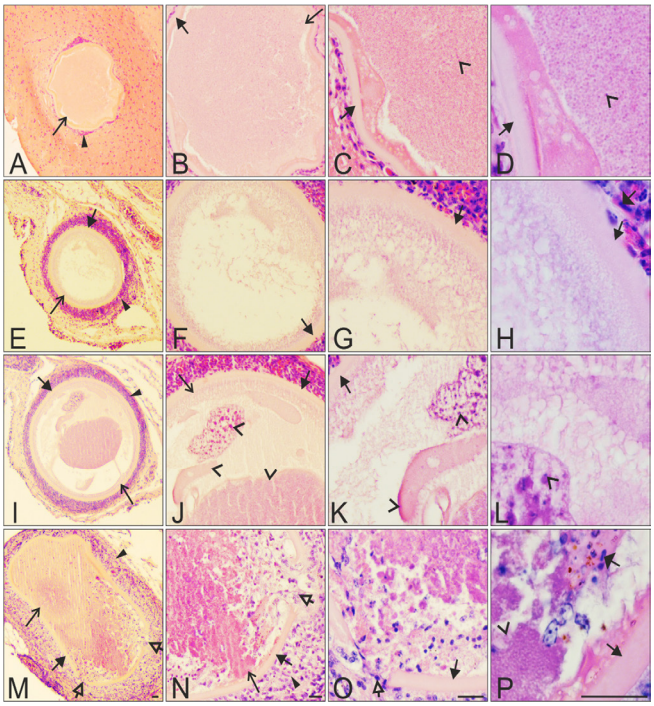

Figure 3. Megalomeronts of *Haemoproteus pastoris* (lineage hLAMPUR01) in the cerebellum of the brain (**A–D**) and esophagus (**E–P**) of naturally infected Common starlings *Sturnus vulgaris*. Note the prominent capsular-like wall covering each megalomeront (**B–P**). The megalomeront in the brain was mature and overfilled with completely developed merozoites

(**C**,**D**). Images E–L show two different histological sections of the same megalomeront, which was cut at different depths; as this megalomeront likely was a roundish body in 3D, the parasite in image I was bigger than in image E due to the location of the former section being closer to the center of the parasite. Note that developing cytomeres were present in section I (located closer to the center of the parasite) but not in section E (located closer to the periphery of the parasite), indicating uneven cytomere location during their development within the same megalomeront. It is possible that nuclear division started first at the center of the roundish megalomeronts (section I), and while the parasite grew, megalomeront division continued until the merozoites reached the extremities of the megalomeronts (section E). Images M–P show a mature ruptured megalomeront containing mature merozoites and infiltration of blood cells inside the megalomeront. Each megalomeront was shown at four different magnifications: A, E, I, M ×100; B, F, J, N ×200; C, G, K, O ×400, and D, H, L, P ×1000. Simple arrows (⟶)—megalomeronts. Filled-black triangle arrows (➔)—capsular-like wall. Contoured-black triangle arrows (➪)—rupture of the capsular-like wall. Flat triangle arrows (➔)—red blood cells inside or outside the megalomeront. Triangle arrowheads (►)—deformed adjacent tissue cells suppressed by the megalomeront. Simple arrowheads (>)—merozoites. All scale bars = 20 μm.

One large, fully mature, and non-ruptured, bean-shaped megalomeront was observed in the pancreas of one bird individual; it measured 800 μm in its maximum length and about 560 μm in its width (Figure 4I–L). This parasite was overfilled with myriads of mature merozoites (Figure 4J–L), pressing against the capsular-like wall, which was up to 8 μm in width.

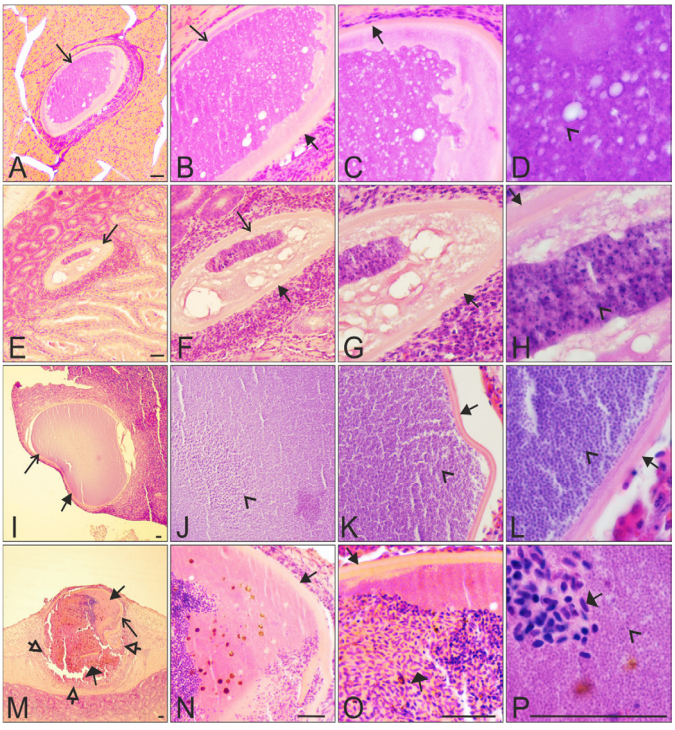

Figure 4. Megalomeronts of *Haemoproteus pastoris* (lineage hLAMPUR01) in the gizzard (**A–D**), the intestine (**E–H**,**M–P**), and the pancreas (**I–L**) of naturally infected Common starlings *Sturnus vulgaris*. Note the prominent capsular-like wall covering each megalomeront (**B**,**C**,**F–H**,**K**,**L**,**N**,**O**), the oval-shape of two megalomeronts (**A**,**E**), and the big size-difference

between developing and mature megalomeronts (**A**,**E** vs. **I**,**M**). Megalomeronts were seen at different stages of development: E–H growing immature, A–D growing maturing, I–L completely mature, and M–P ruptured mature at the stage of degeneration. The megalomeront shown in E-H seemed to present cytomeres at two degrees of maturation, with the more mature merozoites in dark-purple color (**H**) while the rest of the megalomeront remained light pink and showed a less-developed structure; this could indicate an asynchronous development of cytomeres within the same megalomeront. Megalomeronts with a ruptured wall (**M**) showed marked infiltration of blood cells (**M**–**P**), with still some merozoites present (**P**). Each megalomeront is shown at five different magnifications: I, M ×40; A, E, ×100; B, F, J, N ×200; C, G, K, O ×400, and D, H, L, P ×1000. Simple arrows (⟶)—megalomeront. Filled-black triangle arrows (➔)—capsular-like wall. Contoured-black triangle arrows (➢)—rupture of the capsular-like wall. Flat triangle arrows (➔)—red blood cells invading the megalomeront. Simple arrowheads (>)—merozoite. All scale bars = 40 μm.

Several mature ruptured megalomeronts were found (Figures 3M–P, 4M–P, and 5; Table 1). They were mainly big and roundish bodied (570 μm in maximum diameter) with a still-visible capsular-like wall, which measured between 3 and 14 μm in width. However, the capsular-like wall was seen ruptured in one to several places (Figures 3M–O, 4M, and 5D–I), allowing erythrocytes and other cells to enter the megalomeront, while the merozoites leaked out (Figures 3M–O, 4M, and 5D–I). Finally, a structure full of blood corresponding to a former megalomeront, but still surrounded by a capsular-like wall, was observed in several organs (Table 1) and represented a stage of the degeneration of the megalomeront, as all merozoites had already escaped and only the host response was visible (Figure 5A–C). These parasites were mentioned as 'ruptured' in Table 1.

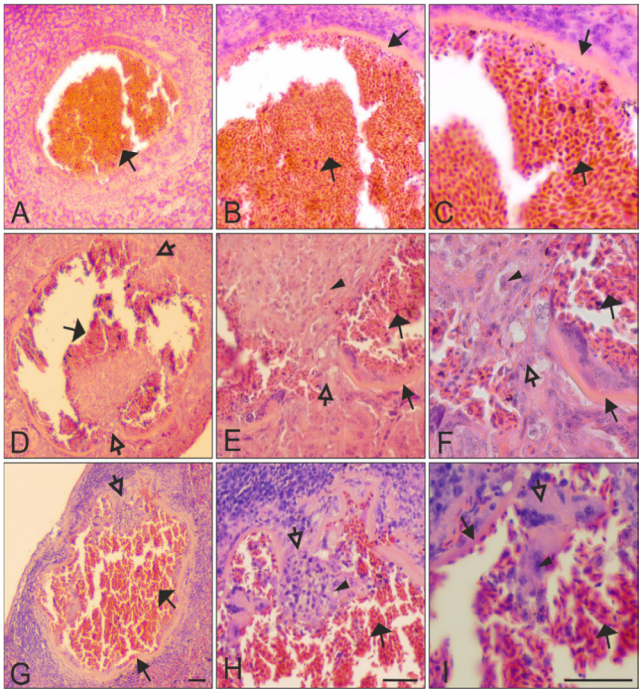

Figure 5. Ruptured mature megalomeronts of *Haemoproteus pastoris* (lineage hLAMPUR01) in the pancreas (**A**–**C**), the intestine (**D**–**F**), and the spleen (**G**–**I**) of naturally infected Common starlings

Sturnus vulgaris. Note that the prominent capsular-like wall was still present after megalomeront maturation and release of merozoites (**B**,**C**,**E–I**), resulting in maintenance of a prominent space, which was invaded with numerous erythrocytes and other blood cells (**A–C** vs. **D–I**). Parasites were shown at three different magnifications: A, D, G ×100; B, E, H ×200, and C, F, I ×400. Filled-black triangle arrows (➔)—capsular-like wall. Contoured-black triangle arrows (➪)—rupture of the capsular-like wall. Flat triangle arrows (➔)—red blood cells invading the megalomeront. Triangle arrowheads (►)—other cells invading the megalomeront. All scale bars = 40 μm.

4. Discussion

The major outcome of this study was the discovery of exo-erythrocytic stages (megalomeronts) of *Haemoproteus* parasites developing in the brain of its avian host (Figure 3A–D). This indicates formerly underestimated pathology during avian haemoproteosis. Former studies observed megalomeronts of *Haemoproteus* species in the heart [11,16,19,20,25,27], intestine [22], kidneys [18,22,26], liver [17,18,26,36], lungs [19,20,26,36], muscles [10,11,13,15,16,19,21,23,27], gizzard [12,20], and spleen [10,26]. In this study, megalomeronts of *H. pastoris* (lineage hLAMPUR01) were observed in nine organs of naturally infected Common starlings, including the brain of one individual (Table 1). This finding is worth attention in future research aiming to better understand mechanisms of virulence during avian haemoproteosis.

It is important to note that the possibility that the found megalomeronts could belong to other blood parasites species can be ruled out due to the following observations. First, 19 Common starlings were examined microscopically, and other blood parasites were not seen, except for *Lankesteralla* sp. in bird no. 3. Second, the primers used for molecular barcoding could detect *Plasmodium* and *Haemoproteus* infections. Only one *Plasmodium relictum* infection was detected by PCR-based testing in one starling (individual 5). The life cycle of *P. relictum* was investigated experimentally, and megalomeronts were absent not only in this malaria parasite species but also in all described *Plasmodium* species in all groups of vertebrates [1]. *Plasmodium* parasites, which were reported before in Common starlings [3], do not develop megalomeronts but meronts [37], the structure and development of which differ from megalomeronts remarkably. *Leucocytozoon* species can be readily distinguished due to the presence of the markedly enlarged host cell nuclei [38,39], which is not the case for *Haemoproteus* species, and this character was absent in all found megalomeronts. Importantly, in all examined *H. pastoris* infected birds, we found the same lineage, morphotype, and megalomeronts of the same morphology. This repetition readily showed *H. pastoris* infection.

It is worth noting that exo-erythrocytic stages have been reported before in the brain of birds during other haemosporidian infections. Several species of *Plasmodium* sp. parasites produce phanerozoites in the brain [1,37,40–42], and some species of *Leucocytozoon* sp. develop megalomeronts in this organ as well [13,43,44]. Interestingly, both during *Plasmodium* and *Leucocytozoon* infections, exo-erythrocytic stages can be found all over the body of hosts as they develop in non-specialized cells, which are also present all over the body, i.e., endothelial cells lining capillaries (*Plasmodium* spp.) and macrophages (*Leucocytozoon* spp.) [13,39,42,43]. If the type of host cells during megalomerogony of *Haemoproteus* infections remains non-identified, it seems to be non-specialized cells as well, as nine different organs were seen to be parasitized during *H. pastoris* infection (Table 1), adding to the already-known parasitized organs for the other *Haemoproteus* species [13,22]. As megalomeronts are big in size and markedly deform the host cells and adjacent organ tissue, morphological characters cannot be used to determine the origin of the host cell. Further targeting studies using histochemistry and immunological methods are needed to answer this question.

The number of *Haemoproteus* species for which megalomeronts have been found, described, and linked to a lineage remains less than 10 (Figure 2) out of more than 150 identified species [2,45]. All studies reported big size of mature megalomeronts (usually >100

µm in biggest diameter) [13,20–26]. Megalomeronts of all described species of haemosporidians were covered with a capsular-like wall, and their maturation included a stage of formation of cytomeres [1,13,18,24,26]. This study supported these observations (Figures 3I–L, 4A–H). Based on these two features, megalomeronts of *Haemoproteus* parasites are similar to megalomeronts of *Leucocytozoon* spp. In both parasite groups, the megalomeronts were covered by a hyaline wall of host origin [25,38], protecting developing parasites from the host immune system [13]. It is easy to distinguish megalomeronts of *Haemoproteus* and *Leucocytozoon* parasites due to presence of the enlarged host-cell nucleus in the latter (also called a central body) [38,39] and its absence in the former organisms [13,25].

The observed growing megalomeronts of *H. pastoris* showed asynchronous development of cytomeres, which were more developed in one part of the megalomeront (Figures 3I–L, 4A–H) while looking less developed in other parts with regard to differentiation of nuclei (Figure 3E–H). This might have been due to different rates of merogony in different cytomere lobules of the parasite. Shown in Figure 3I–L, the parasite showed three different levels of maturation inside the same megalomeront in one section; this was visible due to differing intensity of the nuclear material staining; another section of the same megalomeront did not show cytomeres (Figure 3E–H). Another hypothesis would be that this under-developed part might actually not have been parasite material but belonged to the host cell itself. If we take the example of *H. minutus* investigated with in situ hybridization [25], the paler-outer structure present inside the wall did not react to the parasite probes, indicating its host origin. However, this would need more investigation as no such reports have been addressed for haemosporidians. Both these hypotheses might be tested by the combination of two approaches. First, experimental *Haemoproteus* infection, which would provide an opportunity to access development of megalomeronts in dynamics (for methods see [27,46]), and second, application of specific RNA probes, which would distinguish host and parasite structures (such as in situ hybridization, for methods see [25]).

Similar differences in color and cytomere structure could also be seen in the oval megalomeronts present in the intestine; in this parasite, better-differentiated merozoites were visible in one cytomere and less developed in another one, and their space occupation inside the wall also differed (compare Figure 4A–D vs. Figure 4E–H). It is worth mentioning that the organization of cytomeres within the megalomeront could have been parasite species-specific. Mainly, readily visible differences in cytomere morphology have been observed between the investigated *Haemoproteus* species thus far. Overall, megalomeront structure shows more differences than similarities between *Haemoproteus* species [13,20,21,26]. Among the distinctive features of megalomeronts, the following are readily distinctive: clustering of megalomeronts in tightly closely adjacent groups in *H. minutus* and *H. asymmetricus* [20,25]; development of megalomeronts only in tissues of the skeletal muscles (*H. mansoni*, *H. velans*, *H. syrnii)* and gizzard (*H. sacharovi)* [12,21,23,27]; presence of distinct separately located individuals cytomeres (*H. passeris*) [36]; grouping cytomeres separated from each other by septa (*H.* sp.) [24]; cytomeres interconnected (*H. majoris*) [22,26], or grouped cytomeres in tighter masses (*H. pastoris*) (Figures 3 and 4).

Interestingly, the asynchronous development was seen both within i) the individual megalomeronts in relation to cytomeres formation (see above paragraphs), and within ii) the individual host in relation to different stages of parasite maturation (Table 1). Several stages of megalomeront development were observed in the same host—from growing (Figures 3A–L, 4A–H), to mature (Figure 4I–L), and fully ruptured megalomeronts (Figures 3M–P, 4M–P, and 5A–I). This is similar to the development of *Haemoproteus mansoni* (syn. *Haemoproteus meleagridis*) megalomeronts, which were studied during an experimental infection [27] and were observed from early growing forms to mature and ruptured forms in different individuals of the same host species. Taking into account the four examined naturally infected different Common starling individuals (Table 1), the pattern was similar to these experimental data. Mainly, the completely matured megalomeronts were far bigger than the growing ones (Figures 4I–L vs. 3E–L, 4A–H), and their inside

space was fully packed with mature merozoites (Figure 4I–L); the ruptured megalomeronts had their surrounding wall ruptured in one to several places and its general outline appeared a bit wavier (Figures 3M–P, 4M–P, 5) [10,11,27]. In other words, even within one individual host, different stages of growth of megalomeronts could be found; they did not develop synchronously (Table 1, Figures 3–5). This was also previously observed in *H. majoris*, in which megalomeronts were more mature in the kidneys than in the intestine [22].

Asynchronous development of exo-erythrocytic stages (meronts and megalomeronts) have been reported in many *Plasmodium* and *Leucocytozoon* species due to different times of sporozoite penetration in host cells and also the presence of several generations of merogony [1,47,48]. The latter remains insufficiently investigated in *Haemoproteus* parasites [49]. Due to asynchronous development and maturation of merozoites, gametocytes also develop asynchronously, and this likely is important for transmission and infection of vectors [1].

Research on exo-erythrocytic stages of *Haemoproteus* parasites remains scarce, nonetheless, the available limited data suggest that phylogenetically distant species likely have different megalomeront morphology, as discussed above (Figure 2). However, parasites of well-supported phylogenetic lineage clades have similar megalomeront morphology and location, as is the case in different lineages of *H. majoris* [22,26]. This indicates that phylogenies based on cyt *b* partial genes might be used to predict megalomeront morphology and location in still-non-investigated parasites. Further studies are needed to prove this hypothesis.

The parasitemia intensity in all dissected bird individuals was different and relatively high (Table 1). Interestingly, reports of megalomeronts were not directly related to parasitemia intensity. For example, in bird no. 4 (Table 1), parasitemia was higher than in birds nos. 1–3 but megalomeronts were not seen in no. 4. Furthermore, the four megalomeront-positive individuals had different parasitemia intensity, but megalomeronts were found in a similar number of organs in all birds (three to four positive organs per individual, Table 1). *H. majoris* megalomeronts were found in different host species in two to four different organs [22,26], *H. minutus* in three organs [20], and *H. syrnii* and *H. velans* in one organ [21,23]. These data show that intensity of megalomeronts might have been low and they might have been difficult to find in histological sections even during high parasitemia; this should be taken into consideration during planning of histopathology research using naturally infected birds.

Megalomeronts of *H. pastoris* were mostly roundish, but also showed variability in size and shape, depending on the organ in which they were found and the stage of their development. The majority of observed growing megalomeronts were of a roundish shape (Figure 3A–L), and a few were of an oval-shape, but the latter were only seen in the gizzard and the intestine (Figure 4A–H). This is reminiscent of the young and small *H. majoris* (hPHSIB1) megalomeront found in the intestine of a wood warbler (*Phylloscopus sibilatrix*); it was found to be more oval than roundish in shape [22].

Megalomeronts have thus far always been reported surrounded by a capsular-like wall, which is of host origin [25], but it is still unclear of which host cells and structures it is composed [13,20–26]. The capsular-like wall, which surrounds the megalomeront, seems to be a malleable structure that can extend with the development of the megalomeront, fitting the parasite size and its location in organs, as its thickness was similar within reported variation in all observed megalomeronts of the same species [20,25,26,45]. It also showed a rigidness as it was still present after the maturation and burst of the megalomeront, keeping its place even after rupture (Figures 3–5).

Indeed, the presence of the relatively rigid wall allowed the visualization of the fully ruptured megalomeront, from which merozoites escaped (Figure 3M–P, 4M–P, and 5). These merozoite-empty megalomeronts were full of red blood cells (Figure 5A–I). Due to the capsular-like wall, the ruptured megalomeronts partly maintained their shapes. As the wall surrounding growing megalomeronts is thick, entire, and is of host origin [25],

no inflammatory reaction was observed around the parasites before the burst of the megalomeront. In fact, former studies also did not report the presence of inflammatory reaction around megalomeronts [13,21,24]. However, after the rupture of the wall, the merozoites came into contact with the host tissues, and a host response appeared. The latter was readily visible due to development of bleeding and prominent blood portions filling the megalomeront through the parts where the wall had broken (Figures 3M–P, 4M–P, and 5D–I) [10,11]. Hemorrhage-related processes likely are important pathologies during *H. pastoris* infection and might occur in haemoproteosis caused by other *Haemoproteus* spp. From this point of view, avian haemoproteosis might be similar to *Leucocytozoon (Akiba) caulleryi* hemorrhages caused in domestic chicken [47,50] and, thus, worthy of experimental research. In the case of *H. pastoris*, completely mature and ruptured megalomeronts were found in the brain, the pancreas, the intestine, the esophagus, and the spleen.

The range and timing of megalomeront development in different organs remain to be investigated. It might be that (i) some organs are preferred for megalomeront development at first, and later they develop in other organs, (ii) or they develop faster in some organs, (iii) or the cells they infect are not equally available in all organs, (iv) or other factors which remain unclear. Experimental research would be helpful to answer these questions, but remains rare for *Haemoproteus* spp. [10,27].

Depending on the organ and tissue where the megalomeronts develop, and also the parasite size, we observed a different amount of tissue cells that surround the parasite. These cells were arranged around the capsular-like wall as a thick layer, which was well recognizable in stained histological preparations (Figures 3, 4A,E,I,M, and 5A,D,G). The layer consisted of cells adjacent to the megalomeronts, which were pushed while the megalomeront grew bigger. Few cells seem to be gathered around megalomeronts in the muscles and heart [20,21,23–25], while in parenchyma-rich organs (liver, lungs, spleen, and kidneys) more cells were observed around megalomeronts [22,26]. This showed marked pressure, which megalomeronts produce within organs, and might be important in disease pathology, which needs further research.

This study did not provide certain information about how the reported infections might influence bird health, as the investigated Common starling individuals were wild-caught and dissected within several hours after catching once the parasitemia was confirmed. However, it was clear that the birds were leading active life in wildlife, at least for some time, since they were flying and entered the traps themselves. Presence of high parasitemia and pathological damage in organs by the presence of megalomeronts suggest that parasitized Common starlings might be ill, but still able to fly. Experimental observations are needed for better understanding of pathogenicity during avian haemoproteosis, but such studies are rare [10,12,27]. Some experimental observations showed that birds severely infected with *Plasmodium* parasites often experience sudden mortality: they look active in the evening but are found dead in the morning next day [1,37,40]. Similar events might occur during severe haemoproteosis, particularly during damage of the brain that can cause cerebral ischemia resulting in cerebral paralysis. Except rare observations on dead bird individuals, which were monitored in rehabilitation centers, zoos, and also a limited number of experimental observations [20,21,23,24], the knowledge about what might happen to wild-caught individuals because of haemosporidian infection remains limited. Several studies have reported hemorrhages, multifocal necrosis, and macroscopic organ changes (enlargement, changes of color), which were seen in *Haemoproteus* spp.-infected birds at necropsy [20–24].

The only *Haemoproteus* species recorded in the Common starling was *H. pastoris*, with only one lineage hLAMPUR01 reported (Table 2). This species and lineage have only been found in three species of birds, all belonging to the Sturnidae. Furthermore, only the hLAMPUR01 lineage was found in two of the bird species (Table 2). This indicates that this parasite might be specific to a few species of the Sturnidae. It is possible to speculate that adaptation to develop in birds of the Sturnidae provides opportunities to explore many organs for exo-erythrocytic development of *H. pastoris* (Table 1). If this hypothesis

is correct, the same occupation of many organs might be expected in other host-specific *Haemoproteus* parasite lineages. In this regard, it is worth mentioning that *H. majoris* lineages parasitize several Paridae, Sylviidae, and Turdidae species among other avian hosts, and would thus be of broader specificity than *H. pastoris* [22,26,51]. Megalomeronts of *H. majoris* preferably developed in kidneys in all examined avian hosts [22,26]. It is also worth mentioning that other *Haemoproteus* parasites are found in the same host as *H. majoris* (MalAvi database, [3]), and thus, competition might happen during the development. It seems that a broad vertebrate host specificity of a *Haemoproteus* lineage might be related to a more specialized development of the parasite in certain groups of cells and organs. However, the available information on exo-erythrocytic development remains premature even for preliminary conclusions. Further accumulation of data on exo-erythrocytic development of avian haemoproteids is needed.

Table 2. Molecular records of *H. pastoris* (lineage hLAMPUR01) and non-identified to species level closely related lineage hLAMPUR02 in these avian hosts, according to MalAvi database [3], July 2021.

Parasite Species (Lineage)	Recorded	Bird Species (Family)	Country (Continent)
H. pastoris (hLAMPUR01)	1	*Sturnus vulgaris* (Sturnidae)	Bulgaria (Europe)
	1		Turkey (Asia)
	1		Iran (Asia)
H. pastoris (hLAMPUR01)	1	*Sturnus roseus* (Sturnidae)	Bulgaria (Europe)
H. pastoris (hLAMPUR01)	1	*Lamprotonis pupureiceps* (Sturnidae)	Gabon (Africa)
H. sp. (hLAMPUR02) [a]	1	*Lamprotonis pupureiceps* (Sturnidae)	Gabon (Africa)

[a] Genetic difference between the lineages hLAMPUR01 and hLAMPUR02 is 5 bp or 1.05%.

5. Conclusions

This study discovered and described megalomeronts in *H. pastoris*. For the first time, it was shown that the brain can be parasitized by megalomeronts during avian haemoproteosis. This finding broadens understanding of pathologies caused by avian *Haemoproteus* species and calls for further pathology research during these common and widespread bird infections. Hemorrhage-related processes likely are important pathologies during *H. pastoris* infection and might occur in haemoproteosis caused by other *Haemoproteus* species once megalomeronts mature and burst out merozoites. This issue is related to bird health and worth targeting in experimental research. Asynchronous development is a characteristic feature of *H. pastoris*, with asynchronous development of cytomeres inside individual megalomeronts and different megalomeronts in the same individual host. The mechanism of asynchronous development of cytomeres in individual megalomeronts remains unclear and worthy of attention for better understanding exo-erythrocytic development in haemosporidian parasites. The available information shows that structure of megalomeronts in all investigated *Haemoproteus* species is different, and these differences are supported by phylogenetic analysis, indicating the important value of this character for future taxonomy and *Haemoproteus* parasite biodiversity research.

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Data Availability Statement: The data, including parasite voucher preparations, are available on request from Nature Research Centre, Vilnius, Lithuania.

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PAPER III

Massive infection of lungs with exo-erythrocytic meronts in European robin *Erithacus rubecula* during natural *Haemoproteus attenuatus* haemoproteosis.

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Massive Infection of Lungs with Exo-Erythrocytic Meronts in European Robin *Erithacus rubecula* during Natural *Haemoproteus attenuatus* Haemoproteosis

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Simple Summary: *Haemoproteus* parasites are cosmopolitan bird pathogens belonging to the order Haemosporida (Apicomplexa). A majority of the described species are transmitted by *Culicoides* biting midges, which inject infective stages (sporozoites) in birds during blood meals. The sporozoites initiate tissue merogony, resulting in numerous merozoites, part of which penetrate red blood cells and produce blood stages (gametocytes), which are infective for vectors. The blood stages of *Haemoproteus* parasites have been relatively well-investigated, although tissue stages and patterns of their development remain unidentified in the majority of *Haemoproteus* species. Nevertheless, they often damage various organs which makes them important for bird health. This study contributes new knowledge about tissue merogony of *Haemoproteus attenuatus*, which parasitize birds of the Muscicapidae. Naturally infected European robins *Erithacus rubecula* were caught in Lithuania during autumnal migration. Parasites were identified using morphological features of gametocytes and DNA sequence analysis. Organs of infected birds were examined using histological methods. Tissue stages (meronts) were present only in the lungs, where they were numerous and markedly varied in shape, size and maturation stage. Description of meronts was provided and molecular phylogenetic analysis identified closely related lineages that could present similar exo-erythrocytic development in lungs. Lung damage caused by meronts of *H. attenuatus* and closely related lineages is worth attention due to their possible implications on a bird's health.

Abstract: *Haemoproteus* species are widespread avian blood parasites belonging to Haemoproteidae (Haemosporida). Blood stages of these pathogens have been relatively well-investigated, though exo-erythrocytic (tissue) stages remain unidentified for the majority of species. However, recent histopathological studies show that haemoproteins markedly affect bird organs during tissue merogony. This study investigated the exo-erythrocytic development of *Haemoproteus* (*Parahaemoproteus*) *attenuatus* (lineage hROBIN1), the common parasite of flycatchers (Muscicapidae). Naturally infected European robins *Erithacus rubecula* were examined. Parasite species and lineage were identified using microscopic examination of blood stages and DNA sequence analysis. Parasitaemia intensity varied between 0.8 and 26.5% in seven host individuals. Organs of infected birds were collected and processed for histological examination. Tissues stages (meronts) were seen in six birds and were present only in the lungs. The parasites were usually located in groups and were at different stages of maturation, indicating asynchronous exo-erythrocytic development. In most parasitized individuals, 100 meronts were observed in 1 cm^2 section of lungs. The largest meronts reached 108 µm in length. Mature meronts contained numerous roundish merozoites of approximately 0.8 µm in diameter. Megalomeronts were not observed. Massive merogony and resulting damage of lungs is a characteristic feature during *H. attenuatus* infections and might occur in related parasite lineages, causing haemoproteosis.

Keywords: haemosporidian parasites; *Haemoproteus*; birds; exo-erythrocytic stages; meronts; lung damage

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1. Introduction

Avian haemosporidians (Haemosporida, Apicomplexa) are cosmopolitan parasites [1], which infect representatives of the majority of bird orders and are particularly prevalent in terrestrial bird populations [2], while, with rare exceptions, they are less often found in birds inhabiting marine and costal environments [3]. These pathogens are obligate heteroxenous. Species belonging to genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* are transmitted exclusively by blood-sucking dipterans (Insecta, Diptera). *Plasmodium* spp. are transmitted by mosquitoes (Culicidae), *Haemoproteus* (*Haemoproteus*) spp. by hippoboscid flies (Hippoboscidae), *Haemoproteus* (*Parahaemoproteus*) spp. by biting midges (Ceratopogonidae), *Leucocytozoon* (*Leucocytozoon*) spp. by simuliid flies (Simuliidae) and *Leucocytozoon* (*Akiba*) spp. by biting midges (Ceratopogonidae) [2]. Sporozoites, which are the infective stage for avian hosts, are injected during the vector's blood meal and are transported in the blood stream to tissues of various organs where they initiate exo-erythrocytic development (meronts and/or megalomeronts). Meronts are usually relatively small (predominantly < 100 µm in length) thin-walled structures, which can be readily distinguished from megalomeronts, which are larger structures (predominantly > 100 µm in length) with a thick capsular-like wall [4]. Numerous unicellular merozoites develop in meronts and megalomeronts. Mature merozoites are released into the circulation, inhabit red blood cells and produce gametocytes, which are infective for vectors [2].

Gametocytes of haemosporidians are relatively well-studied life cycle stages, which are easy to access for microscopic examination and PCR-based research due to their presence in the peripheral blood circulation. However, tissue stages of haemosporidians are more difficult to access because this requires the dissection of bird organs and application of histopathological techniques [2]. Knowledge about exo-erythrocytic development of avian haemosporidian parasites remains scarce, particularly in *Haemoproteus* species. These haemosporidians have been formerly considered to be relatively benign avian parasites and have thus attracted insufficient attention in avian medicine and avian pathology research [4]. However, recent molecular studies combined with histopathology observations have proved that some *Haemoproteus* species cause disease and even mortality in non-accustomed avian hosts due to pathologies initiated by megalomeronts [5–10]. These findings called for further research of the exo-erythrocytic development of haemoproteids, particularly their virulence during development in specific tissues. *Haemoproteus* spp. exo-erythrocytic stages have been found in lungs, liver, spleen, kidneys, heart, brain, bone marrow, proventiculus, gizzard, caecum, tongue, intestine and skeletal muscles [4,11–13]. It is possible that many other organs and tissues can be involved in tissue merogony during haemoproteosis. Further studies are needed for a better understanding of the development of haemosporidian parasites in vertebrates, an issue which is directly related to bird health [4].

The aim of this study was to contribute to the characterization of the exo-erythrocytic development of *Haemoproteus attenuatus* (cytochrome *b* -cyt *b*- lineage hROBIN1) in naturally infected European robins *Erithacus rubecula*. We initiated this study due to a note in an unpublished histological observation [14], which reported the presence of meronts of *H. attenuatus* in lungs and spleen of one individual of European robin sampled during spring migration on the Baltic Sea coast. Valkiūnas [2] described this finding briefly, however, the available data about tissue merogony of *H. attenuatus* remained limited to the single observation, and the pathogen genetic lineage remained non-identified. Because *H. attenuatus* is prevalent in flycatchers of the Muscicapidae [2], we extended the observation on tissue stages in the naturally infected juvenile and adult European robins. Numerous meronts were found in lungs of parasitised birds, indicating a pattern in exo-erythrocytic development during *H. attenuatus* haemoproteosis. Phylogenetic analysis identified closely related lineages of haemoproteids inhabiting the Muscicapidae birds, suggesting a possibly similar pattern of exo-erythrocytic development of these pathogens.

2. Materials and Methods

2.1. Study Area and Sample Collection

Seven *Haemoproteus* parasite-infected European robins were caught at the Ornithological station Ventes Ragas (55°20′38.93″ N, 21°11′34.05″ E), Lithuania during autumnal migration in September 2020. Large Rybachy-like traps, zigzag and funnel traps were used for catching the birds. Among them were: 5 juveniles, 1 adult and 1 individual of unidentified age. Blood was sampled from the branchial vein and used for blood film preparation and storage in SET-buffer (0.05 M tris, 0.15 M NaCl, 0.5 M EDTA, pH 8.0) for further molecular analysis. Blood films were air dried, fixed in methanol (1 s) and stained using a 10% Giemsa solution for on-site microscopic examination following [2]. During the fieldwork, blood film microscopic examination was used to determine the presence of the parasite in the circulation, as well as preliminary species identification. SET-buffer stored blood was used later in the laboratory for parasite lineage determination (see description below). Seven *H. attenuatus*-positive birds were euthanized by decapitation, according to permits and their organs were dissected for histological examination.

2.2. Blood and Histological Samples

In the laboratory, blood films were stained using a 10% buffered Giemsa solution for one hour [2]. The brain, heart, intestine, kidneys, liver, lungs, pectoral muscles, spleen, and stomach were processed for histological investigation. These organs were fixed in 10% neutral formalin in the field and processed in the laboratory for long-term storage by being embedded in paraffin blocks. From each block of paraffin-embedded-organ, 4 µm sections were prepared, mounted on glass slides, air-dried and stained with haematoxylin-eosin (H&E) following standard protocols [2,15].

An Olympus BX51 light microscope equipped with an Olympus DP12 digital camera and Olympus DP-SOFT imaging software was used to examine stained blood films and histological sections. Blood films were examined for 15 min at low magnification (×400) to find infected birds. If parasites were present, 100 microscope fields were scanned at high magnification (×1000) to estimate relative infection intensity (number of parasites in 100 fields), and then parasitemia (number of parasites in 2000–10,000 erythrocytes, depending on relative infection intensity) according to [16]). Parasite species was determined at high magnification according to [2]. Histological preparations were examined at medium (×400) and high (×1000) magnification. If exo-erythrocytic meronts were found, they were examined at different magnifications (100, 200, 400 and ×1000) to identify their morphological traits and location in the organs. Exo-erythrocytic stages were then measured using ImageJ 1.53a software (National Institutes of Health, Bethesda, MD, USA, https://imagej.nih.gov/ij/USA; accessed on 10 October 2021) [17].

Voucher parasite preparations containing gametocytes (accession numbers of blood slides 49361NS-49363NS) and tissue meronts (accession numbers of histological sections of lungs 49364NS-49366NS) were deposited at Nature Research Centre, Vilnius.

2.3. DNA Extraction, PCR and Sequencing

DNA was extracted from blood samples stored in SET-buffer using an ammonium acetate protocol [18]. The samples were diluted to a concentration of 25 ng/µL for PCR work. A standard nested PCR protocol was applied to identify the lineage in each individual bird infected with *H. attenuatus*. The primers HaemNFI/HaemNR3 and HaemF/HaemR2, as well as the parameters of PCR, were the same as those described in the original protocol [19,20]. Positive (*Haemoproteus* sp.) and negative (nuclease-free water) controls were used as tests for possible false amplifications. PCR products were run on a 2% agarose gel to check for positive amplifications, which were sequenced from 3′ end with Big Dye Terminator V3.1 Cycle Sequencing Kit and ABI PRISMTM 3100 capillary sequencing robot (Applied Biosystems, Foster City, CA, USA). The resulted 479 bp sequences of the cytochrome *b* mitochondrial gene were checked using the SnapGene Viewer 5.2.4 software (Insightful Science, San Diego, CA, USA, www.snapgene.com; accessed on 10 October 2021)

for presence of double peaks (a test for possible co-infections) and quality. The identification of lineage was carried out by BLAST-searches in MalAvi database [21] and GenBank with Megablast algorithm (www.ncbi.nlm.nih.gov/genbank/; accessed on 10 October 2021). The obtained DNA sequence information was compared with the results of the parasite microscopic identification.

2.4. Phylogenetic Analysis

A phylogenetic tree of 36 cytochrome *b* lineages (479 bp) of avian haemosporidians was constructed using Bayesian inference in MrBayes 3.2.7 software (University of Rochester, Rochester, NY, USA; Evolutionary Biology Centre, Uppsala, Sweden) [22], which is available at the CIPRES Science Gateway [23]. Thirty lineages of *Haemoproteus*, five of *Plasmodium*, and 1 of *Leucocytozoon* (as outgroup) were used. These were DNA sequences of parasite species, which are closely related to *H. attenuatus* (the so-called parasites of a *Haemoproteus balmorali* group [2]), and other *Haemoproteus* species for which exo-erythrocytic stages have been described previously. Analysis was performed with two runs of four chains each, a 25% burn-in, 15 million generations and saving 15,000 trees. The quality of the analysis (effective sampling size, traces of the two runs, and burn-in) was examined using the Tracer 1.7.1 software (University of Edinburgh, Edinburgh, Scotland; University of Auckland, Auckland, New Zealand; University of California, Los Angeles, CA, USA) [24]. Majority rule consensus tree and the posterior probabilities were visualized using the FigTree 1.4.4 software (University of Edinburgh, Edinburgh, Scotland) [25]. Mitochondrial DNA sequences were aligned with MUSCLE [26] in Mesquite 3.61 software (The University of British Columbia, Vancouver, BC, Canada; Oregon State University, Corvallis, OR, USA) [27]. We performed the molecular analysis using a general time reversible model with gamma distribution and a proportion of invariable sites (GTR + G + I) as obtained from jModelTest 2 software (University of Vigo, Vigo, Spain; University of A Coruña, Coruña, Spain) [28]. Genetic distances between the different lineages of a *H. attenuatus* and *H. balmorali* clade (Figure 1, clade Aa) were estimated using the Jukes-Cantor model, with uniform substitution rate among sites in the Mesquite 3.61 software.

3. Results

3.1. Molecular Analysis

Single infection of *H. attenuatus* cyt *b* lineage hROBIN1 was found in the seven European robins during PCR screening. Phylogenetic analysis clustered the lineage hROBIN1 with three lineages of *H. balmorali* (hCOLL3, hSFC1 and hLULU1) in a well-supported clade (posterior probability of 1), suggesting close evolutionary relationships (Figure 1). Genetic distances between the lineages of *H. attenuatus* and *H. balmorali* clade (Figure 1, clade Aa) were small, ranging from 0.2% (hROBIN1-hLULU1) to 3.2% (hROBIN1-hSCF1).

3.2. Blood Stages

Parasitemia intensity varied between 0.8 and 26.5% in seven examined European robins (Table 1). Observed gametocytes belonged to *H. attenuatus* (Figure 2A–C). This finding corresponded with the obtained DNA sequence information because the lineage hROBIN1 was detected. Only single infections were seen in blood films and electropherograms. Distinct characteristic features of this parasite species were readily visible (Figure 2A,C), particularly in microgametocytes. Among these features, the attenuated dumbbell-shaped growing gametocytes (Figure 2A,B) and the presence of prominent roundish volutin granules should be pointed out. Volutin granules were predominantly gathered close to the poles of gametocytes (Figure 2C).

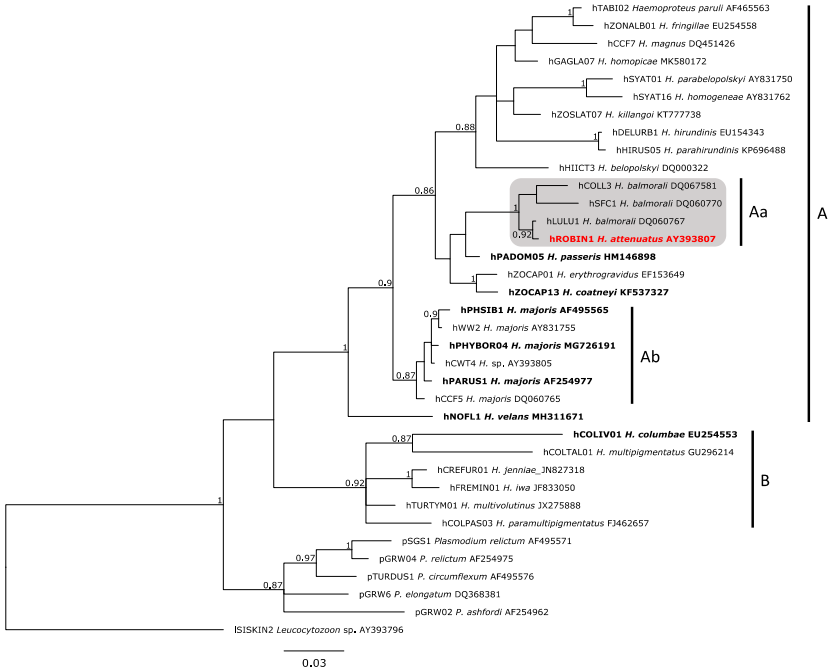

Figure 1. Bayesian phylogeny based on cytochrome *b* gene fragments (479 bp) of 36 lineages of avian haemosporidian parasites, including 30 lineages of *Haemoproteus*, 5 of *Plasmodium* and one of *Leucocytozoon*, which was used as outgroup. Parasite lineages were shown, followed by species names and DNA sequence GenBank accession numbers. A gray box indicates the clade containing *Haemoproteus attenuatus* (hROBIN1). Vertical bars show species of subgenera *Parahaemoproteus* (**A**) and *Haemoproteus* (**B**) as well as lineages of *Haemoproteus balmorali* (**Aa**) and *Haemoproteus majoris* (**Ab**) groups (see Discussion for explanation). *Haemoproteus* species, for which exo-erythrocytic development was formerly reported, were shown in bold font. Species investigated in this study are given in bold font and a red color. Numbers on the nodes represent posterior probabilities. Scale bar indicates the number of expected substitutions per site.

Table 1. Age, parasitemia and histological observations of seven European robins *Erithacus rubecula* infected with *Haemoproteus attenuatus* (cytochrome *b* lineage hROBIN1).

Sample No.	Age	Parasitemia (%)	Meronts in Lung Tissue	Meront Length (μm), Min–Max (*n*)	Enlarged Spleen	Darkened		Figure
						Spleen	Liver	
1	Juvenile	0.8	+	17.1–49.8 (7)	–	+	–	
2	Juvenile	0.95	–	–	+	+	–	
3	Juvenile	1.85	+	5.8–77.58 (25)	–	–	–	Figure 2D,H,I and Figure 3A–C
4	Juvenile	2.9	+	40.4–99.8 (5)	+	+	+	Figure 2M–O
5	Juvenile	23.6	+	20.6–28.5 (2)	–	+	+	
6	Adult	10.45	+	108.7 (1)	–	+	–	
7	Unknown	26.5	+	9.46–94.27 (14)	–	–	–	Figure 2E–G,J–L

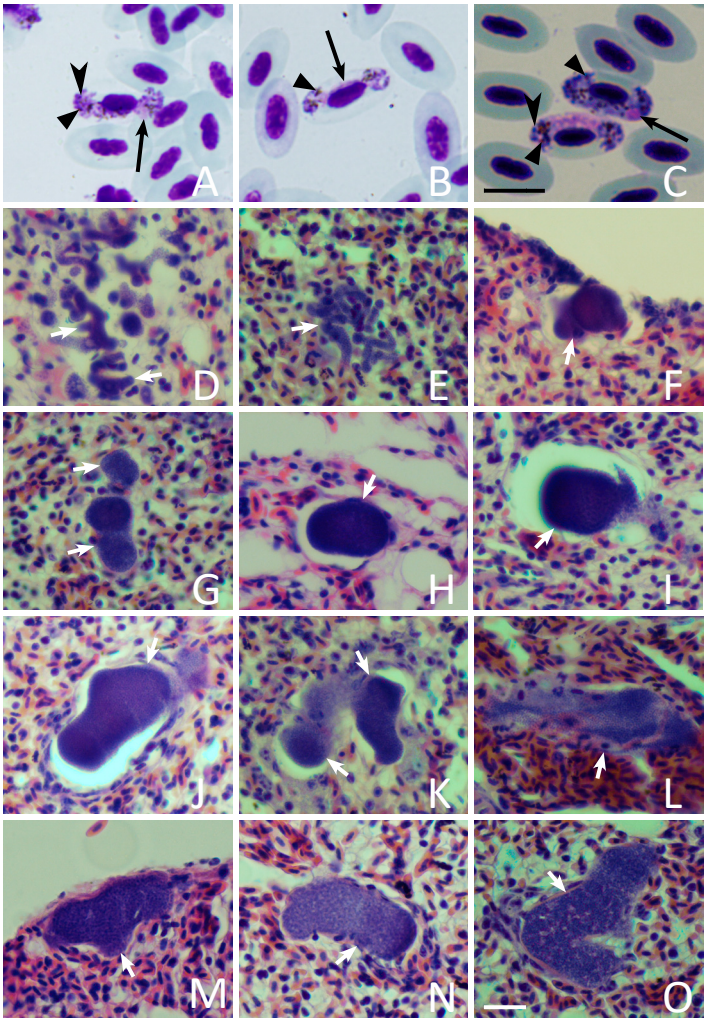

Figure 2. The same section of the lungs of European robin *Erithacus rubecula*, with meronts of *Haemoproteus attenuatus* (cytochrome *b* lineage hROBIN1) shown at different magnifications, ×100 (**A**), ×200 (**B**), ×400 (**C**), and ×1000 (**D**). A simple white arrow shows the same meront in this group of parasites. Location of meronts in groups is a characteristic feature of this infection (**A**,**B**). Note that meronts are markedly different in size and shapes, but the majority did not exceed 50 µm at the biggest diameter. Scale bar is 50 µm for all images.

3.3. Exo-Erythrocytic Stages

Megalomeronts were not seen. Meronts were found in six birds (4 juveniles, 1 adult, 1 of unidentified age; Table 1) and were present only in the lungs, where they were usually located in groups and occurred at different stages of maturation, indicating an asynchronous exo-erythrocytic development (Figure 2D–O). Meronts were not observed in one juvenile bird, in which parasitemia intensity was relatively low (0.95%). Some dissected individuals presented spleen (1 adult, 4 juveniles) and liver (2 juveniles) blackness, as well as spleen enlargement (2 juveniles; Table 1).

Meronts were of markedly variable shapes and sizes. The largest one reached 108 µm at the greatest length, but the majority were smaller and usually did not exceed 50–70 µm (Figure 2D–I and Figure 3A–D). Variously shaped parasites were seen (i.e., roundish, oval, worm-like and branching; Figure 2D–O and Figure 3A–D). The elongated worm-like meronts predominated at early stages of development, suggesting that initial development occurs in the capillaries of the lungs. Available data show that the growing parasites first follow the shape of capillaries, extending along them and assuming thin elongate forms (Figure 2D,E). Then, the capillaries are blocked and deformed by growing meronts, which assume various shapes when completely grown (Figure 3A–D). The nucleus of the host cell was not seen near meronts. Vacuoles were also invisible in growing meronts, but vacuole-like spaces appeared in completely mature meronts at stage of their rupture (Figure 3D). Cytomeres were not seen at any stage of meronts growth. Mature meronts (Figure 2G–O) contained a homogenous mass of numerous roundish merozoites of approximately 0.8 µm in diameter.

Meronts were covered by a thin, often hardly visible envelope, lacking a capsular-like wall. However, largest meronts markedly pushed surrounding lung tissues, resulting in the pressure of connective fibres and the appearance of an interrupted (not entirely) thick envelope-like structure (Figure 2O). Due to interruption, this structure differed markedly from a typical entire capsular-like wall, which always develop around the megalomeronts. The interrupted thick-walled structures were absent around small meronts, which push the lung cells lightly (Figure 2F–I). In other words, the development of a thick interrupted envelope around *H. attenuatus* meronts was a function of the parasite size.

The number of meronts observed in the 1 cm^2 section of lungs ranged from one in the least infected lungs to 100 in the most intensively infected. The largest meronts markedly pushed the surrounding lung tissues, likely resulting in a blockage of circulation in the capillaries and a deformation of alveoli (Figure 3A–D). Inflammatory reaction was not seen around the growing and maturing meronts (Figure 3A–D), but slight infiltration of blood cells was seen inside and around the largest ruptured meronts (Figure 2K,L), indicating the presence of haemorrhagic symptoms. Some cellular infiltrations were visible in the alveoli septae (Figure 2L). Furthermore, the air spaces were also seen occluded to an almost pneumonic level (Figure 2K,O), although infiltration with white blood cells was not visible.

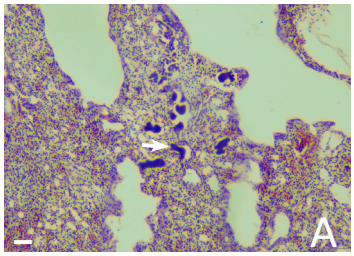

Figure 3. *Cont.*

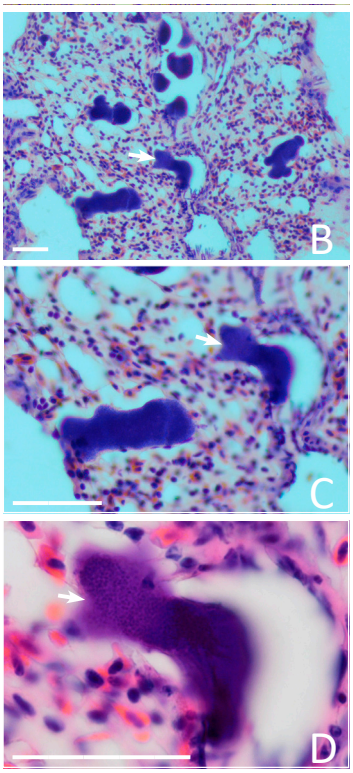

Figure 3. Gametocytes (**A**–**C**) and lung meronts (**D**–**O**) of *Haemoproteus attenuatus* (cytochrome *b* lineage hROBIN1) in naturally infected European robin *Erithacus rubecula*: (**A**, **B**) growing macrogametocyte (**A**) and microgametocyte (**B**); (**C**) fully grown macrogametocyte (top) and microgametocyte (bottom); (**D**,**E**) groups of numerous early developing meronts; (**F**–**O**) variously shaped maturing meronts, including those at the stage of differentiation of merozoites (**J**–**O**). Note that growing gametocytes of this parasite are markedly attenuated (**A**,**B**), which is particularly visible in microgametocyte (**B**). Prominent roundish volutin granules are well-visible in gametocytes (**A**,**C**), particularly microgametocytes, in which they gather close to the poles of the gametocytes (**C**). The largest meronts markedly push surrounding lung tissues resulting in appearance of interrupted thick envelope-like structures, which resemble a wall around some meronts (**O**), but such structures were absent around smaller meronts, which push lung cells less (**F**–**I**). Simple long black arrows—gametocyte nuclei; simple black arrowheads—volutin granules; triangle black arrowheads—pigment granules; simple short white arrows—meronts at different stages of their development. Scale bars: black—10 μm (for images **A**–**C**), white—20 μm (for images **D**–**O**).

4. Discussion

The main result of this study is the proof that the lungs are an important site for exo-erythrocytic development of *H. attenuatus* (hROBIN1). Meronts were observed only in the lungs. This study supported the observation by Iezhova [14] who reported morphologically similar meronts in lungs of one individual European robin infected with non-identified lineage of *H. attenuatus*. Because several *Haemoproteus* lineages have been detected in European robin (MalAvi database, Lund University, Lund, Sweden. Available online: http://130.235.244.92/Malavi; accessed on 10 October 2021), it was important to specify certain host-parasite association for this tissue stage in our study. Photographs of *H. attenuatus* meronts (Figure 2D–O and Figure 3A–D) were published for the first time. Meront morphology and localization was the same in both studies, indicating a pattern of exo-erythrocytic development of this parasite, which multiplies preferably in the lungs. Interestingly, meronts were seen in all dissected birds, except for one individual, in which parasitemia was one of the lowest (0.95%), indicating that the lung meronts may be rare and difficult to find during low parasitemia. However, the individual with the lowest parasitemia (0.8%) presented lung merogony as well as a darkened spleen.

The lineage hROBIN1 has been reported in three *Culicoides* spp. and nine bird species belonging to six families in Europe, Africa and Russia (Table 2). However, sporozoites were not observed in two *Culicoides* spp., and gametocytes of *H. attenuatus* were only seen in the blood of four bird species. In other words, presence of invasive stages (sporozoites in vectors and gametocytes in avian hosts) were not documented, meaning that some reports might be abortive infections of *H. attenuatus* [4].

Table 2. Hosts and locations where *Haemoproteus attenuatus* (cytochrome *b* lineage hROBIN1) have been reported.

Host Order	Host Family	Host Species	Location [a]	Reference
Diptera	Ceratopogonidae	*Culicoides festivipennis* [b]	Lithuania	Bernotienė et al., unpublished [d]
		C. obsoletus [b]	Lithuania	Bernotienė et al., unpublished [d]
		C. nubeculosus	Lithuania	[29]
Coraciiformes	Alcedinidae	*Alcedo atthis* [b]	Spain	Rojo et al., unpublished [e]
Passeriformes	Certhiidae	*Certhia familiaris* [b]	Sweden	[30]
	Acrocephalidae	*Acrocephalus schoenobaenus* [b]	Sweden	[30]
	Sylviidae	*Sylvia communis* [b]	Sweden	Hellgren et al., unpublished [d]
	Muscicapidae	*Erithacus rubecula* [c]	Bulgaria, Germany, Lithuania, Morocco, NWA, NWI, Portugal, Russia, Serbia, Spain, Sweden	[30–37]
		Luscinia luscinia [c]	Lithuania, Russia, Sweden, Turkey, WGC	[30,32,34,38–41]
		L. megarhynchos [c]	Bulgaria, Germany, TRC	[31,32,34]
		Saxicola rubetra	Nigeria, Sweden, TRC	[30,32,34]
	Turdidae	*Turdus merula* [b]	TRC	[32]

[a] NWA—North West Africa; NWI—North West Iberia; WGC—West Greater Caucasus; TRC—Transcaucasia. [b] Reports were not supported by observation of invasive stages (sporozoites in vectors or gametocytes in birds). These might be abortive infections (dead ends of transmission), particularly because gametocytes of *H. attenuatus* have never been documented in these bird species. [c] Co-infections with *Haemoproteus balmorali* are common in these hosts. This is an obstacle to link observed blood stages and genetic sequence information. [d] NCBI GenBank data. [e] MalAvi database data (MalAvi database. Available online: http://130.235.244.92/Malavi/; accessed on 10 October 2021).

Lungs have been reported as the site of meront location in several species of *Haemoproteus*, including *Haemoproteus nettionis* [42], *Haemoproteus orizivorae* [43], *Haemoproteus balearicae* [44], *Haemoproteus coatneyi* [45], *Haemoproteus columbae*, *Haemoproteus* sp. [46] and *Haemoproteus passeris* (see review in [2]). Interestingly, lung meronts were of similar morphology in all these parasites, and their morphology corresponded to description given in this study. Mainly, all reported lung meronts were of markedly variable sizes, shapes and developed without formation of cytomeres and capsular-like walls. DNA barcoding is available for some of these parasites. The phylogenetic analysis showed that these species are not closely related (Figure 1), probably indicating an independent evolution of the ability to inhabit lung cells in different *Haemoproteus* species.

Interestingly, Iezhova [14] reported numerous meronts of *H. attenuatus* (non-identified lineage) in lungs of an European robin sampled during spring migration, and this study found them in the same host species and organ in big numbers during autumnal migration, indicating that infected birds are present and can be detected for research during the entire period of transmission from spring to autumn in Europe. It is important to note that the presence of parasites in juvenile birds (this study) shows the local infection transmission. This information is worth attention when planning further research of this and related *Haemoproteus* infections in birds. Complete sporogony development of *H. attenuatus* (hROBIN01) occurs in the biting midge *Culicoides nubeculosus*, which might be the natural vector [29]. The same lineage was reported in *Culicoides festivipennis* and *Culicoides obsoletus*, the common biting midges in Europe (Table 2). The closely related parasite *H. balmorali* (an unidentified lineage and the lineage hSFC9) completed sporogony in *Culicoides impunctatus* [47,48]. Reports of *H. attenuatus* (hROBIN01) both in vectors and birds (Table 2) show that the transmission conditions of this infection are present in Europe.

Iezhova [14] found a single meront of *H. attenuatus* in the spleen of a naturally infected European robin, which was sampled during spring migration in May. This season corresponds to a spring relapse-period in haemosporidian parasites in Europe [2]. These data suggest that *H. attenuatus* might occasionally develop in the spleen. The latter organ might be the site of localization of persisting tissue stages, which are responsible for spring relapses, but remain insufficiently investigated in avian *Haemoproteus* parasites. Meronts in the spleen were not observed in this study, which was the autumn sample and is thus not related to spring relapse. The host–parasite association '*H. attenuatus* (hROBIN1) and European robin' can be used for a deeper investigation of persistence in avian haemosporidians.

Infections detected in our study most likely corresponds to recently gained infections. Most of the infected individuals were juveniles (Table 1), meaning that they got infected on the same year of sampling. Due to the fact that only one adult bird was examined, it is not possible to make any conclusions about the influence of age of the host on merogony and pathologies found in spleen and liver, neither on the size and number of meronts or parasitemia. Nevertheless, our results suggest that even in cases of low parasitemia, alterations in spleen and liver may be present, which could have a negative implication on the host's health.

Megalomeronts were not observed in this and Iezhova's [14] studies, indicating that they might be absent during exo-erythrocytic development of *H. attenuatus*. The limited histological observations from natural infected birds that are available so far have reported the presence of only meronts [14,42–46,49,50], only megalomeronts [11,51–55] and both of these exo-erythrocytic stages [56–71] in different *Haemoproteus* species. A fundamental issue in biology of avian *Haemoproteus* parasites remains unresolved. Mainly, it remains unclear whether or not megalomeronts develop in all *Haemoproteus* species. In other words, it remains uncertain whether the development of both meronts and megalomeronts is an obligatory character of these parasites on a genus level. It might be that megalomeronts do not occur in some *Haemoproteus* species. It is possible that a certain sequence of occurrence during the exo-erythrocytic development (presence of meronts or megalomeronts, or both) might be a function of pathogen species or even certain host-parasite association. For

example, the same isolate of *Leucocytozoon simondi*, a common haemosporidian parasite of anseriform birds, developed megalomeronts in ducks, but not in geese [2]. Megalomeronts are easy to visualise in histological sections due to their big size [11]. Meronts of some *Haemoproteus* parasites are small (close to 10 µm in diameter), contain few merozoites and are similar to meronts of *Plasmodium* spp. both by morphology and localization in organs [50], so they might be difficult to find and identify using microscopic examination of H&E stained histological sections, particularly during low intensity. Molecular diagnostic tools (chromogenic in situ hybridization) are essential in future studies of exo-erythrocytic stages, and they have already been developed [52,53]. Further targeting research is needed to better understand patterns of tissue merogony in haemosporidians. This is an important issue for current parasitology research because tissue merogony, particularly development of megalomeronts, is associated with gross pathology and is a severe, sometimes even lethal avian disease [51].

It is important to note that *H. attenuatus* (hROBIN1) is closely related to several lineages of *H. balmorali*, which also parasitize birds of the Muscicapidae (Figure 1, clade Aa). Morphological data are in accordance with these phylogenetic data. Mainly, gametocytes of these parasites share the same distinct species characters, particularly due to the presence of volutin granules of similar size, shape and location (Figure 2A–C). Recent studies show that closely related parasites, which partial cyt *b* gene sequences cluster in well-supported clades, also have tissue stages of a similar morphology and localization. For example, this is the case in different lineages of *H. majoris* (Figure 1, clade Ab), which different lineages produce megalomeronts of a similar morphology and localization in different avian hosts [11,52]. Therefore, closely related lineages of *H. attenuatus* and *H. balmorali* (Figure 1, clade Aa), which have similar gametocytes, might also present similar merogony in the lungs. In other words, when planning examination of tissue merogony of different *H. balmorali* lineages, the lungs are worth to be targeted as an important site of location of meronts first of all. This conclusion is in accordance with observation of Iezhova [14] who reported a single lung meront of non-identified lineage of *H. balmorali* in spotted flycatcher *Muscicapa striata*. Further research into a better understanding of the possible predictability of molecular phylogenies in determination of tissue merogony in haemosporidian parasites is of practical value because it might not only speed up research on this subject, but may also help to predict pathological changes in organs based solely on DNA sequence information.

This study provides limited information on the possible influence of tissue merogony on birds because it was based only on the material collected in naturally infected hosts that were euthanized (Table 1). The birds were caught in stationary traps, meaning that they were actively flying. However, the massive infection of lungs and blockage of capillaries, as well as occlusion of alveoli by tissue meronts (Figure 3), should be related to lung disfunction and a lowering of the competitive ability of intensively infected individuals. This health state is difficult to measure and correlate with bird survival without targeting experimental observations combined with field studies. Some cellular infiltrations were visible in the alveoli septae (Figure 2L). Furthermore, the air spaces were also seen occluded looking like light pneumonic degree (Figure 2K,O), although infiltration with white blood cells was not visible. High parasitemia can also hardly be neutral for the hosts, in which an enlargement and blackness of the liver and spleen was visible, indicating gross pathological changes in parenchymal organs ([2] this study). It is possible that migration behavior, which is a key feature in this host species, might be affected or even disrupted in heavily infected bird individuals. It is worth noting that former studies suggested that high *Haemoproteus* sp. parasitemia is associated with a decrease in the accumulation of migratory fat, which is the main energetic material for migrating birds [2]. Further experimental studies are needed for a better understanding of the pathologies that occur during haemoproteosis, which remains a neglected avian disease.

5. Conclusions

Lungs were the primary site of exo-erythrocytic development during *H. attenuatus* (hROBIN1) infection. Massive infection of lungs by meronts was determined and described in naturally parasitized birds. Megalomeronts were not observed and might not occur in this parasite during development in European robins and closely related Muscicapidae species, however, further research is needed to answer this question. Available observations and phylogenetic analysis suggested that the lineages of the *H. balmorali* group might have a similar pattern of tissue merogony, as is the case in the closely related *H. attenuatus*. Lung pathology due to the occlusion of lung capillaries and air spaces is worth attention in relation to bird health during *H. attenuatus* and related *Haemoproteus* infections.

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Data Availability Statement: Data from this study can be available on request.

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PAPER IV

Exo-erythrocytic development of two *Haemoproteus* species (Haemosporida, Haemoproteidae), with description of *Haemoproteus dumbbellus*, a new blood parasite of bunting birds (Emberizidae).

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Exo-erythrocytic development of two *Haemoproteus* species (Haemosporida, Haemoproteidae), with description of *Haemoproteus dumbbellus,* a new blood parasite of bunting birds (Emberizidae)

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ABSTRACT

Avian haemosporidians are widespread parasites categorized into four families of the order Haemosporida (Apicomplexa). Species of the subgenus *Parahaemoproteus* (genus *Haemoproteus*) belong to the Haemoproteidae and are transmitted by *Culicoides* biting midges. Reports of death due to tissue damage during haemoproteosis in non-adapted birds have raised concerns about these pathogens, especially as their exo-erythrocytic development is known for only a few *Haemoproteus* spp. More research is needed to better understand the patterns of the parasites' development in tissues and their impact on avian hosts. Yellowhammers *Emberiza citrinella* (Emberizidae) and common house martins *Delichon urbicum* (Hirundinidae) were screened for *Haemoproteus* parasites by microscopic examination of blood films and PCR-based testing. Individuals with single infection were selected for histological investigations. H & E-stained sections were screened for detection and characterization of the exo-erythrocytic stages, while chromogenic *in situ* hybridization (CISH) and phylogenetic analysis were performed to confirm the *Haemoproteus* origin and their phylogenetic relationships. *Haemoproteus dumbbellus* n. sp. was discovered in *Emberiza citrinella* single-infected with the lineage hEMCIR01. Meronts of *H. dumbbellus* n. sp. developed in various organs of five of six tested individuals, a pattern which was reported in other *Haemoproteus* species clustering in the same clade, suggesting this could be a phylogenetic trait. By contrast, in *Delichon urbicum* infected with the *Haemoproteus* lineage hDELURB2, which was linked to the more distantly related parasite *Haemoproteus hirundinis*, only megalomeronts were found in the pectoral muscles of two of six infected individuals. All exo-erythrocytic stages were confirmed to be *Haemoproteus* parasites by CISH using a *Haemoproteus* genus-specific probe. While the development of meronts seems to be typical for species of the clade containing *H. dumbbellus*, further investigations and data from more species are needed to explore whether a phylogenetic pattern occurs in meront or megalomeront formation.

1. Introduction

Genetic lineages of avian haemosporidians (Haemosporida, Apicomplexa) have been recorded in 2195 bird species all over the world, except for Antarctica (MalAvi database, Lund University, Lund, Sweden, https://130.235.244.92/Malavi/ last access in September 2022) (Bensch et al., 2009; Clark et al., 2014). The parasites classify into four families – Plasmodiidae, Haemoproteidae, Leucocytozoidae and Garniidae (Valkiūnas, 2005). Parasites of the Haemoproteidae belong to the genus *Haemoproteus*, which consists of two subgenera – *Haemoproteus* and *Parahaemoproteus* –, species of which are transmitted by louse flies (Hippoboscidae) and biting midges (Ceratopogonidae, subgenus *Culicoides*), respectively (Valkiūnas, 2005; Chagas et al., 2019; Valkiūnas and Atkinson, 2020). After injection of sporozoites by the vectors into susceptible avian hosts, exo-erythrocytic stages develop (Valkiūnas and Iezhova, 2017). Within these stages, merozoites form, which later

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infect host erythrocytes to become gametocytes, the infective stage for vectors (Valkiūnas, 2005).

The Haemoproteidae family is rich in species number, with 177 *Haemoproteus* spp. described based on the gametocytes' morphology and peculiarities of their influence on host cells (Valkiūnas and Iezhova, 2022). At the same time, 1905 genetic lineages of these parasites have been reported, but only 159 (or 8%) of them were linked to 76 morphospecies (MalAvi database, last access in September 2022 (Bensch et al., 2009)), suggesting an even higher species diversity. Opposed to the rapidly increasing data on the genetic diversity of these parasites due to the implementation of PCR-based methodologies, many aspects about the development of these parasites in their avian hosts remain unknown for most of the described species. This is particularly true for the exo-erythrocytic stages of the parasites, which are known only fragmentarily for less than 30 species of *Haemoproteus* (Valkiūnas and Iezhova, 2017; Himmel et al., 2019, 2021; Ilgūnas et al., 2019, 2022; Ortiz-Catedral et al., 2019; Duc et al., 2020, 2021; Hernández-Lara et al., 2021; Yoshimoto et al., 2021; Harl et al., 2022).

Exo-erythrocytic stages (or tissue stages) of *Haemoproteus* parasites did not attract much attention until the recent reports of deaths due to haemoproteosis (Ortiz-Catedral et al., 2019) in non-adapted birds, which usually do not contract the parasites of certain lineages and thus are less able to cope with them. Such infections often result in severe disease and even mortality due to damage of organs by the parasites' exo-erythrocytic stages (Ortiz-Catedral et al., 2019).

Haemoproteus parasites can develop into two different types of exo-erythrocytic stages – meronts and megalomeronts. Meronts usually do not exceed 70 μm in length (Hernández-Lara et al., 2021); they are often of irregular form and covered by a thin eosinophilic wall. Megalomeronts have been reported to be up to 800 μm in size (Duc et al., 2021); they are often of roundish or oval form and covered by a thick capsular-like wall of host origin. Recent studies on exo-erythrocytic stages of *Haemoproteus* spp. reported the presence of either only meronts (*Haemoproteus attenuatus* (Hernández-Lara et al., 2021)) or only megalomeronts (*Haemoproteus minutus, Haemoproteus asymmetricus, Haemoproteus majoris, Haemoproteus syrnii, Haemoproteus* sp. (Himmel et al., 2019; Ilgūnas et al., 2019, 2022; Ortiz-Catedral et al., 2019; Yoshimoto et al., 2021)) in the investigated avian hosts. Among these parasites, only two species were reported to develop tissue stages in more than one host species, i. e. *H. syrnii* megalomeronts in two host species (*Strix aluco* and *Strix uralensis* (Ilgūnas et al., 2022)), and *H. majoris* megalomeronts in three host species (*Parus major, Turdus pilaris,* and *Phylloscopus sibilatrix* (Ilgūnas et al., 2019; Duc et al., 2020)). In older literature, four *Haemoproteus* species – *Haemoproteus passeris*, *Haemoproteus handai*, *Haemoproteus mansoni* and *Haemoproteus columbae* – were reported to develop both types of exo-erythrocytic stages, albeit not in the same host individuals and without molecular support (G. Valkiūnas, pers. comm.) (Peirce, 1976; Burtikashvili, 1978; Miltgen et al., 1981; Atkinson et al., 1986, 1988; Earle et al., 1993; Peirce et al., 2004; Valkiūnas and Iezhova, 2017). Unfortunately, experimental studies of the exo-erythrocytic development of *Haemoproteus* parasites are scarce (Atkinson et al., 1986, 1988). Due to the limited information on the merogonic development for the majority of described *Haemoproteus* spp., it remains elusive whether the formation of meronts and megalomeronts reflect functional or developmental adaptations or represent phylogenetically informative traits of the parasites on species levels. These data are necessary to better understand the patterns of development of tissue stages in *Haemoproteus* spp. and to explore whether it is possible to predict the exo-erythrocytic development of closely related parasites using phylogenetic approaches. This, in turn, could help to predict in which organs and how tissue stages could develop in natural and non-adapted hosts during haemoproteosis and then, possibly to plan research on the development of treatment measures.

The present study aimed to investigate the exo-erythrocytic merogony of two *Haemoproteus* parasite lineages, for which tissue stages have not been described: hEMCIR01, a lineage commonly found in *Emberiza citrinella* (MalAvi database, last access in September 2022, (Bensch et al., 2009)) but not yet attributed to morphospecies, and hDELURB2, a putative lineage of *Haemoproteus hirundinis* based on high similarity with *H. hirundinis* hDELURB1 (Chagas et al., 2019) and often detected in *Delichon urbicum* (MalAvi database, last access in September 2022, (Bensch et al., 2009)). Samples collected from *E. citrinella* and *D. urbicum* were investigated for single infections with hEMCIR01 and hDELURB2, respectively, and the development of their exo-erythrocytic stages were examined and discussed in regard to their phylogenetic relationship with other avian haemoproteids. Furthermore, blood stages of these parasites were morphologically characterized, and the lineages attributed to their morphospecies.

2. Materials and methods

2.1. Sample collection

Yellowhammers *E. citrinella* and common house martins *D. urbicum* were collected in the ornithological station Ventė Cape (55°20′38.93″N, 21°11′34.05″E, see https://www.vros.lt), Lithuania, in May of 2017, 2018, 2019, and 2021, using mist nets and a large stationary trap ('Rybachy' type trap). Blood samples were obtained from the brachial vein. From each bird, blood films were prepared, and approximately 20 μL of blood was collected in SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH = 8.0) and stored at −20 °C. The blood films were fixed in methanol, stained with Giemsa and screened microscopically for the presence of haemosporidian parasites using a standard protocol for the detection of avian haemosporidians (Valkiūnas, 2005). Six birds of each species with single infections of *Haemoproteus* parasites as determined by blood film examination, were euthanized, and their organs (brain, heart, lungs, trachea, oesophagus, gizzard, intestine, liver, spleen, kidneys, reproductive organs, and pectoral and leg muscles) were collected for histological examination. In addition to the *E. citrinella* collected in Lithuania, tissue samples of two *E. citrinella* were retrieved from the archive of the Institute of Pathology at the University of Veterinary Medicine Vienna, Austria. These two birds were submitted for post-mortem examination in August 2004, and their organs collected and fixed in neutral buffered formalin for histological examination. Samples of the brain, liver, and spleen were frozen and stored at −20 °C for molecular analysis. Blood films were not available for these two individual specimens.

2.2. Parasitaemia, prevalence and parasite morphology

Blood films were screened using a BX41 (Olympus, Tokyo, Japan) light microscope to determine the infection status of the collected birds and the prevalence of the parasite (percentage of birds infected with the target parasites species out of all collected individuals of the same host species). Blood films were analyzed for 15–20 min at medium magnification (400×) followed by examination of 100 microscope fields at high magnification (1000×). For dissected individuals with available blood films, the intensity of parasitaemia was determined by counting the number of parasites per 1000 erythrocytes or per 10,000 erythrocytes in case of low parasitaemia (Godfrey et al., 1987); measurements and images of gametocytes were taken using an Olympus DP12 digital camera and the Olympus DP-SOFT software. The standard range of *Haemo-*

proteus parasite characters were used for morphological characterization of gametocytes and their host cells for a new *Haemoproteus* sp. (Valkiūnas, 2005).

2.3. Histology and chromogenic in situ hybridization (CISH)

Tissue samples were fixed in 10% neutral buffered formalin, dehydrated in increasing ethanol concentrations (70–100%), clarified in xylene, and embedded in paraffin wax. Histological sections of 2–3 μm were prepared from all collected organs, stained with haematoxylin & eosin staining (H & E), and screened for exo-erythrocytic stages at magnifications of 100x and 200x using Olympus BX41 and B51 light microscopes equipped with Olympus DP12 or UC90 digital cameras, respectively (Olympus, Tokyo, Japan). Higher magnifications (400×, 1000×) were used for taking pictures of exo-erythrocytic stages, using the Olympus image softwares DP-SOFT or cellSens Entry. Acquired photographs were adjusted for brightness and contrast and assembled in CorelDraw 2019 (RRID:SCR_014235, https://www.coreldraw.com/en/). Based on the photographs, measurements of exo-erythrocytic stages were taken using the ImageJ-based imaging processing package Fiji (Image J 1.53c, National Institutes of Health, Bethesda, MD, USA, downloaded at https://imagej.net/software/fiji accessed on August 24 2022) (Schindelin et al., 2012).

In parallel to H & E-stained histological preparations, chromogenic *in situ* hybridization (CISH) using a *Haemoproteus* genus-specific probe (Haemo18S) targeting the *18S* ribosomal RNA of the parasites (Himmel et al., 2019) was conducted on at least one section per organ per individual according to previously described protocols (Dinhopl et al., 2011; Himmel et al., 2019). A sample of one *E. citrinella* individual from Vienna included in this study was previously used for testing the specificity and sensitivity of the Haemo18S probe (Himmel et al., 2019).

2.4. DNA extractions, PCRs, and sequencing

For molecular characterization of parasites detected in the blood films, DNA extraction and PCR screening were performed. DNA extractions of the SET buffer-stored blood samples were done following the ammonium acetate protocol (Richardson et al., 2001). DNA extractions of frozen tissue samples of two *E. citrinella* were performed using the DNeasy Blood & Tissue Kit (QIAGEN, Venlo, Netherlands) following the manufacturer's protocol with the modification of performing two elution steps, each with 100 μl instead of a single elution. The second eluate was used for PCR.

Molecular screening for avian haemosporidians was performed using a nested PCR protocol, which amplifies a 478 bp section of the cytochrome b (*cytb*) gene (Bensch et al., 2000; Hellgren et al., 2004). The outer primer pair HAEMNFI/HAEMNR3, and the inner primer pairs HAEMF/HAEMR2 and HAEMFL/HAEMR2L were applied to detect *Haemoproteus* and *Plasmodium*, and *Leucocytozoon* parasites, respectively. Amplification of parasite DNA using the protocol of Hellgren et al., (2004) was unsuccessful in four of six *D. urbicum* samples, so they were additionally screened with the nested PCR protocol described in (Beadell et al., 2004; Hellgren et al., 2004; Duval et al., 2007; Pérez-Rodríguez et al., 2013) using the primer pairs PLAS1F/HAEMNR3 and 3760F/HAEMJR4, which detect parasites from all three genera. PCR profiles for both nested PCRs were kept as per original protocols, including negative (distilled water) and positive (previously determined *Plasmodium/Leucocytozoon* infected sample) controls. PCR products were examined on 2% agarose gels. Successfully amplified fragments were prepared for Sanger bi-directional sequencing with a Big Dye Terminator V3.1 Cycle Sequencing Kit and ABI PRISM™ 3100 capillary sequencing robot (Applied Biosystems, Foster City, CA, USA), or were sent for bi-directional sequencing to Microsynth (Microsynth, Vienna, Austria).

Obtained sequence chromatograms were analysed using the softwares Geneious Prime 2022.0.2 (Dotmatics, Auckland, New Zealand, https://www.geneious.com), and Bioedit (https://bioedit.software.informer.com/) (Hall, 1999). All sequences were subjected to BLAST search in the MalAvi database, last access in April 2022 (Bensch et al., 2009) and NCBI GenBank (National Library of Medicine, Bethesda, Maryland, https://www.ncbi.nlm.nih.gov/genbank/, last access in April 2022) to identify detected lineages.

2.5. Phylogenetic analysis

A Bayesian phylogenetic tree was calculated using only lineages which were identified to species level, including 34 *Haemoproteus* lineages from the subgenus *Parahaemoproteus*, three lineages from the subgenus *Haemoproteus*, and one lineage of *Leucocytozoon*, which was used as outgroup. All sequences were retrieved from the GenBank database, using their common lineage names from the MalAvi database. The model GTR+I+G was used after checking for the best-fit model in jModeltest-2.1.10 (Guindon and Gascuel, 2003, Darriba et al., 2012) with AIC, AICc, BIC and DT. MrBayes plugin v3.2.6 (Huelsenbeck and Ronquist, 2001) was run in Geneious for 5 million generations, sampled every 100th generation, and discarding the first 25% trees as a 'burn-in' period for the consensus tree.

3. Results

3.1. Parasite identifications and parasitaemia

Six *D. urbicum* and six *E. citrinella* showed single *Haemoproteus* infections both by microscopic examination and PCR-based testing (Table 1).

Gametocytes found in *D. urbicum* displayed characteristics of *H. hirundinis* (Fig. 1A-F; see description below), and the intensity of parasitaemia in *H. hirundinis*-infected birds ranged from 0.04% to 0.53% (Table 1).

Gametocytes present in the blood films of *E. citrinella* displayed unique characters which correspond to a new species (Fig. 2A-P) and were described below. The parasitaemia ranged from 0.26% to 1.04% in different infected individuals of *E. citrinella* (Table 1).

3.2. Lineages and phylogenetic analysis

Molecular analysis of the partial *cytb* sequences revealed single infections for all 14 individuals investigated. *Delichon urbicum* were all infected with the *Haemoproteus* lineage hDELURB2, and *E. citrinella* were all infected with the lineage hEMCIR01 (Table 1). It is important to note, that the lineage hDELURB2 was amplified in only two of six *D. urbicum* samples using the primers HAEMNFI/HAEMNR3-HAEMF/HAEMR2, while the primers PLAS1F/HAEMNR3-3760F/HAEMJR4 successfully amplified hDELURB2 in all six samples. All sequences were deposited in GenBank under the accession numbers **OQ361943-OQ361953, MN025423, MK330150**, and **MK330152**.

Phylogenetically, hDELURB2 was closely associated with the *H. hirundinis* lineage hDELURB1, differing by 7 bp or 1.5% in the barcoding region, while hEMCIR01 clustered with *Haemoproteu* ssp. (hROFI1, 9 bp difference), *Haemoproteus tartakovskyi* (hSISKIN1, 13 bp difference), *Haemoproteus cyanomitrae* (hCYAOLI03, 13 bp difference), and *H. passeris* (hPADOM05, 12 bp difference), among others (Fig. 3). With regard to the species for which exo-erythrocytic stages have been described (presented in bold letters

Table 1
Molecular and morphological identification of parasites detected with location of their exo-erythrocytic stages in *Emberiza citrinella* and *Delichon urbicum*.

Individual	Host species	Collection date	Origin	Parasitaemia (%)	*cytb* lineage	Parasite species	Location (and type) of exo-erythrocytic stages
AH0608	*Emberiza citrinella*	2004-08-17	Austria	n/a	hEMCIR01	*Haemoproteus dumbbellus*	Lungs, gizzard (meronts)
AH0611	*E. citrinella*	2004-08-17	Austria	n/a	hEMCIR01	*H. dumbbellus*	Heart, lungs (meronts)
556/17R	*E. citrinella*	2017-05-15	Lithuania	0.54	hEMCIR01	*H. dumbbellus*	Not available
579/17R	*E. citrinella*	2017-05-16	Lithuania	0.26	hEMCIR01	*H. dumbbellus*	Not available
19/18R	*E. citrinella*	2018-05-16	Lithuania	0.69	hEMCIR01	*H. dumbbellus*	Lungs (meronts)
305/19R	*E. citrinella*	2019-05-11	Lithuania	0.35	hEMCIR01	*H. dumbbellus*	Not found
242/21R	*E. citrinella*	2021-05-17	Lithuania	0.98	hEMCIR01	*H. dumbbellus*	Heart, liver, lungs, leg muscle, gizzard (meronts)
349/21R	*E. citrinella*	2021-05-19	Lithuania	1.04	hEMCIR01	*H. dumbbellus*	Heart, liver, lungs, leg muscle, gizzard, brain (meronts)
337/17R	*Delichon urbicum*	2017-05-09	Lithuania	0.04	hDELURB2	*Haemoproteus hirundinis*	Not found
79/18R	*D. urbicum*	2018-05-18	Lithuania	0.2	hDELURB2	*H. hirundinis*	Not found
82/18R	*D. urbicum*	2018-05-18	Lithuania	0.51	hDELURB2	*H. hirundinis*	Not found
329/21R	*D. urbicum*	2021-05-19	Lithuania	0.53	hDELURB2	*H. hirundinis*	Pectoral muscle (megalomeronts)
323/21R	*D. urbicum*	2021-05-19	Lithuania	0.34	hDELURB2	*H. hirundinis*	Pectoral muscle (megalomeronts)
340/21R	*D. urbicum*	2021-05-19	Lithuania	0.53	hDELURB2	*H. hirundinis*	Not found

Cytb, cytochrome b gene; n/a, not available.

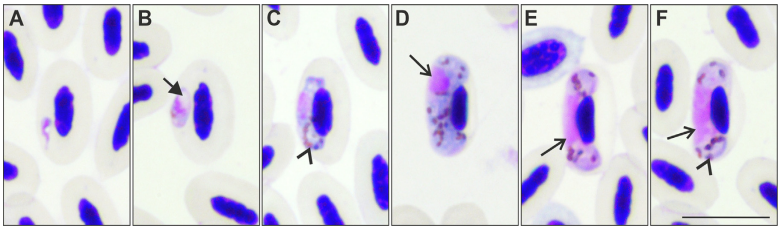

Fig. 1. Gametocytes of *Haemoproteus hirundinis* (lineage hDELURB2) from the blood of a common house martin *Delichon urbicum*. Developmental stages are (A, B) young gametocytes (Note the presence of a vacuole in young gametocytes in (B) as indicated by the arrow); (C) growing, and (D) fully grown macrogametocytes; (E) growing, and (F) fully grown microgametocytes. Parasite nuclei in D-F are indicated by arrows. The arrowheads indicate pigment granules. Scale-bar = 10 μm.

in the tree), the lineage hEMCIR01 was most closely associated with *H. attenuatus* and *H. passeris*, both of which were reported to form meronts (Peirce, 1976, Hernández-Lara et al., 2021). *Haemoproteus hirundinis* hDELUBR2 clustered with species for which exo-erythrocytic stages have not been described yet (e. g. *Haemoproteus lanii*, *Haemoproteus homopicae*, and others) (Fig. 3).

3.3. Description of *Haemoproteus dumbbellus (lineage hEMCIR01)*

Haemoproteus (Parahaemoproteus) dumbbellus n. sp.

Type host: Yellowhammer *E. citrinella* (Passeriformes, Emberizidae).

Barcoding DNA sequence: Mitochondrial *cytb* lineage hEMCIR01 (478 bp, GenBank accession numbers **OQ361943-OQ361948**, **MK330150**, **MK330152**).

Additional hosts: the barcoding lineage was recorded in three bird species: *Emberiza cirlus* (Emberizidae), *Phylloscopus trochilus* (Phylloscopidae) and *Sula nebouxii* (Sulidae) (Dimitrov et al., 2010; Levin et al., 2011; Mata et al., 2015; Ellis et al., 2020); however, these studies do not mention the presence of gametocyte stage in these avian hosts.

Type locality: Ventė Ragas Ornithological station, Lithuania (55°20′38.93″N, 21°11′34.05″E).

Site of infection: Gametocytes develop in mature erythrocytes. Exo-erythrocytic stages (meronts) were seen in the lungs, heart, leg muscle, brain, liver, and gizzard of the host.

Prevalence: Eight of 18 individuals examined (44%).

Type specimens: Hapantotypes (gametocytes, *E. citrinella*, sampled in 2018.05.16, 2021.05.17, 2021.05.19 Ventė Cape, Lithuania, coll. Mikas Ilgūnas, accessions nos. 49446NS, 49460NS, 49469NS; exo-erythrocytic stages: 2004.07.30, Güssing, Burgenland, Austria, coll. Tanja Himmel, accessions nos. 49454NS – 49457NS; 2021.05.17, 2021.05.19, Ventė Cape, Lithuania, coll. Mélanie Duc, accessions nos. 49462NS, 49463NS, 49471NS – 49476NS) were deposited to the Nature Research Centre (NRC), Vilnius, Lithuania.

Parahapantotypes (gametocytes, *E. citrinella*, 2018.05.16, 2021.05.17, 2021.05.19, Vente Cape, Lithuania, coll. Mikas Ilgūnas, accessions nos. 49447NS, 49461NS, 49470NS; exo-erythrocytic stages: 2004.07.09, 2004.07.30, Güssing, Burgenland, Austria, coll. Tanja Himmel, accessions nos. 49452NS, 49453NS, 49458NS, 49459NS) were deposited to the NRC. Parahapantotypes (gametocytes, *E. citrinella*, 2021.05.17, 2021.05.19, Ventė Cape, Lithuania, coll. Mikas Ilgūnas, accessions nos. G466262, G466267, G466273; exo-erythrocytic stages: 2004.07.09, Güssing, Burgenland, Austria, coll. Tanja Himmel, accessions nos. G466263 – G466266, 2021.05.17, Ventė Cape, Lithuania, coll. Mélanie Duc, accessions nos. G466268 – G466272) were deposited to the Queensland Museum, Queensland, Australia.

Additional material: Voucher preparations from *E. citrinella* sampled at the type locality: gametocytes (accession nos. 49450NS, 49451NS) and histological sections (accession nos. 49464NS – 49468NS, 49477NS – 49480NS) were deposited to the NRC.

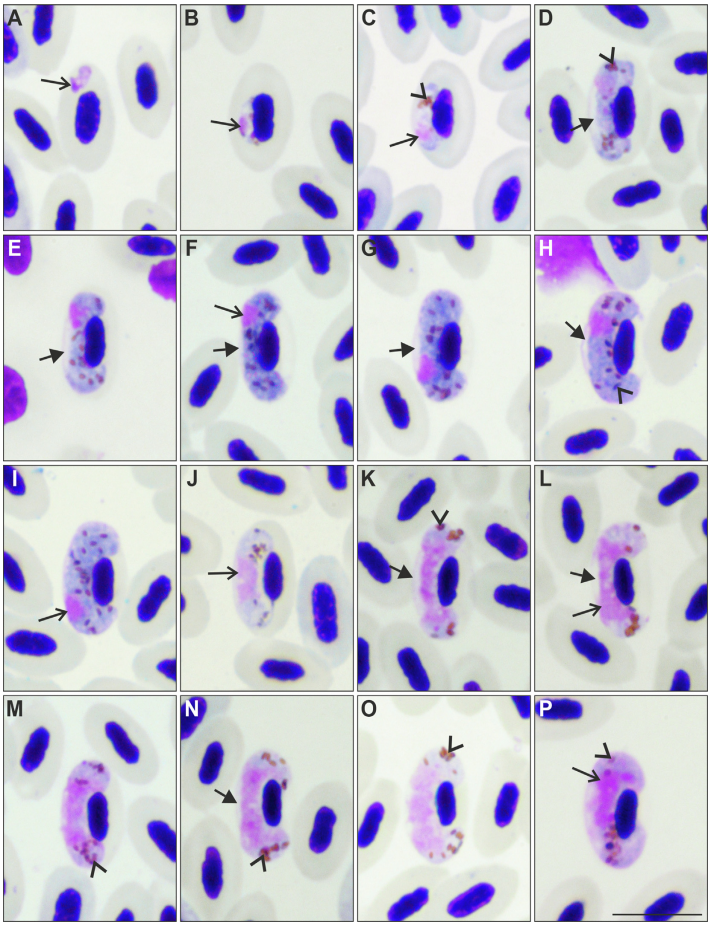

Fig. 2. Gametocytes of *Haemoproteus dumbbellus* n. sp. (lineage hEMCIR01) from the blood of a yellowhammer *Emberiza citrinella*. Developmental stages are (A-C) young gametocytes; (D-I) macrogametocytes; (J-P) microgametocytes. The following forms can be distinguished among them: (D, E) growing macrogametocytes, (F, G) advanced macrogametocytes, (H, I) fully grown macrogametocytes, (J, K) growing microgametocytes, (L-M) advanced microgametocytes, and (N-P) fully grown microgametocytes. Note that early gametocytes (A) do not adhere to the erythrocyte nuclei, but all other blood stages (B-P) adhere to them. The long arrow indicates the parasite nucleus and the arrowheads indicate pigment granules. An unfilled space (indicated by the short arrow in D-H, K, L, N) is present between gametocytes and the envelope of erythrocytes from the stage of developing gametocytes to the stage of fully grown gametocytes. This gives the parasite a dumbbell-like shape at most stages of growth, which is a characteristic feature of this parasite species. Note that this space often maintains in fully grown gametocytes (H, N), a rare character in *Haemoproteus* species. Fully grown gametocytes fill erythrocytes till the poles; they enclose the nuclei of erythrocytes with their ends but do not encircle them completely (H, I, O, P). The macrogametocyte nucleus is subterminal in position; it does not adhere to the erythrocyte nucleus (F-I). Scale-bar = 10 μm.

Etymology: The species name derives from the English word "dumbbell". It reflects the dumbbell-like shape of advanced gametocytes due to the presence of a readily visible space between the gametocytes and envelope of infected erythrocytes (Fig. 2D-I). The dumbbell-like form is present at most stages of gametocyte growth, including the fully grown gametocytes – a rare character in avian haemoproteids.

Young gametocytes (Fig. 2A-C): Earliest forms were usually located in a subterminal to terminal position in the infected erythrocytes. They do not adhere to the host cell nuclei, nor to the erythrocyte envelope (Fig. 2A). As the parasite develops, gametocytes closely adhere to the nuclei of erythrocytes at their lateral side and extend longitudinally along the nuclei, but the young growing forms still do not adhere to the erythrocyte envelope

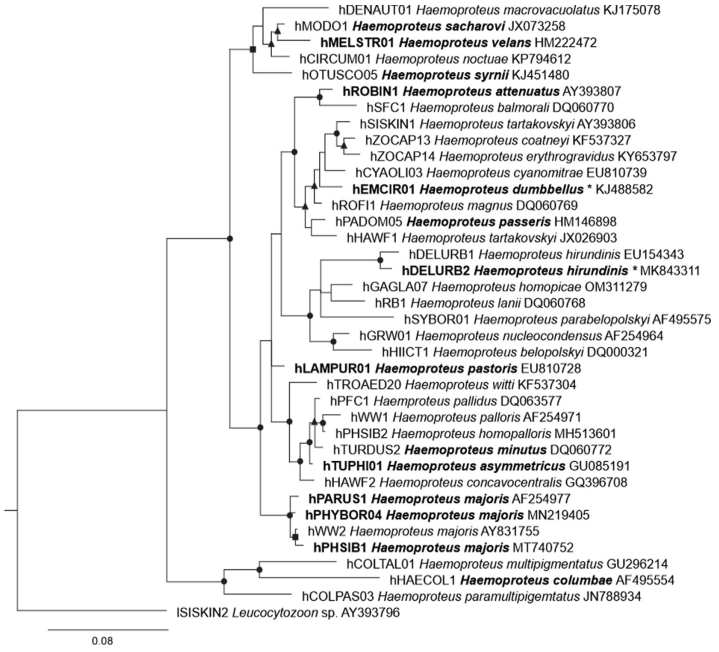

Fig. 3. Bayesian phylogenetic tree of partial cytochrome *b* sequences of 37 *Haemoproteus* spp. lineages and one *Leucocytozoon* sp. lineage as outgroup. Parasites were represented by MalAvi lineage names (Bensch et al., 2009), followed by their species names and GenBank sequences accession numbers. Bold font indicates species (and lineage when known) for which exo-erythrocytic stages (meronts or megalomeronts) have been described. Parasites from this study indicated with an asterisk. Posterior probabilities (PP) are provided with symbols: triangles, PP 0.7–0.8; squares, PP 0.8–0.9; and circles, PP 0.9–1.

(Fig. 2B, C). The gametocyte cytoplasm stains unevenly (Fig. 2C); nuclei are prominent and of irregular shape; pigment granules are well-visible and usually grouped (Fig. 2C); outline is usually even. The influence on host cell is not pronounced.

Macrogametocytes (Fig. 2D-I, Table 2): The cytoplasm is homogenous in appearance and usually does not contain readily visible vacuoles; volutin granules are not seen. Gametocytes grow around the nuclei of infected erythrocytes, they slightly enclose the nuclei with their ends, but never encircle the nuclei completely, leaving a portion of the cytoplasm unoccupied by gametocytes (Fig. 2D-I). Advanced growing gametocytes adhere to the envelope of erythrocytes only by their ends (Fig. 2D, E). An unfilled space between the erythrocyte's envelope and the gametocyte is present and readily visible at all stages of gametocyte growth. As a result, the growing and fully grown gametocytes assume a readily visible dumbbell-like shape, which is a characteristic feature of this species development (Fig. 2D-H). The largest fully grown gametocytes, however, were occasionally seen being nearly appressed to the erythrocyte envelope, resulting in a poorly visible space between the gametocytes and the envelope of erythrocytes, partly losing their dumbbell shape (Fig. 2I). Medium (Fig. 2F, G) and fully grown gametocytes (Fig. 2H, I) fill erythrocytes up to their poles. The parasite nucleus is compact, strictly subterminal in position, variable in form and is located close to the erythrocyte envelope. Pigment granules are roundish or oval, mostly of medium size (0.5–1 µm), usually randomly scattered throughout the cytoplasm. The outline of gametocytes is predominantly even. Nuclei of infected erythrocytes are slightly displaced laterally (Fig. 2D-I, Table 2).

Microgametocytes (Fig. 2J-P, Table 2): The general configuration is as for macrogametocytes, with the usual sexual dimorphism for haemosporidians, which are the pale staining of the cytoplasm, the large diffuse nuclei, and the grouping of pigment granules close to the gametocyte ends. Dumbbell-like shape is readily visible in growing gametocytes (Fig. 2J-L, N), but is often hardly visible in some fully grown gametocytes (Fig. 2O, P). Advanced non-dumbbell shaped microgametocytes (Fig. 2P) are more numerous than in macrogametocytes but remain the minority among all seen microgametocytes. The cytoplasm is usually paler stained at the gametocyte portion adhering to the erythrocyte envelope, when gametocytes were non-dumbbell shaped (Fig. 2P).

Exo-erythrocytic stages (Fig. 4A-T): Exo-erythrocytic meronts were observed in the lungs, heart, gizzard, liver, leg muscle, and brain of infected birds, with the number of affected organs ranging among individuals from one to six (Table 1, Fig. 4). Among all individuals, the lungs were most commonly parasitized, while other organs were less often affected. In the lungs, numerous meronts were disseminated over the section and often clustered in small groups (Fig. 4F, G, J, N-S). The meronts varied in shape, size, and stage of maturation, indicating asynchronous development. They were either roundish or of irregular form (Fig. 4A, B, F-J, O-T), sometimes worm-like or branching meronts, following the shape of blood capillaries (Fig. 4L, C-E, M, N), suggesting localization in

Table 2
Morphometry of host cells and fully grown gametocytes of *Haemoproteus dumbbellus* n. sp. (lineage hEMCIR01) from the blood of the yellowhammer *Emberiza citrinella*. Measurements for length and width are in micrometres (µm), and area is µm^2.

Features	Measurement (µm) (mean ± S.D.)
Uninfected erythrocytes	
Length	10.6–12.5 (11.4 ± 0.3)
Width	5.7–7.0 (6.4 ± 0.1)
Area	48.9–66.0 (57.2 ± 20.3)
Uninfected erythrocytes nucleus	
Length	4.7–6.0 (5.3 ± 0.1)
Width	2.0–2.7 (2.3 ± 0.03)
Area	8.0–13.0 (10.1 ± 1.3)
Macrogametocytes	
Infected erythrocyte	
Length	10.7–13.1 (12.0 ± 0.3)
Width	5.7–7.9 (7.1 ± 0.3)
Area	57.8–76.9 (66.8 ± 28.4)
Infected erythrocyte nucleus	
Length	4.6–6.5 (5.3 ± 0.2)
Width	2.1–2.8 (2.4 ± 0.03)
Area	8.6–12.1 (10.4 ± 0.9)
Gametocyte	
Length	13.6–17.2 (15.4 ± 1.0)
Width	2.3–4.1 (3.4 ± 0.2)
Area	37.6–50.9 (43.4 ± 14.8)
Gametocyte nucleus	
Length	2.1–3.0 (2.5 ± 0.1)
Width	1.2–2.5 (1.9 ± 0.1)
Area	2.3–5.9 (4.1 ± 0.8)
Pigment granules	13.0–17.0 (14.5 ± 2.0)
NDR	0.5–0.9 (0.7 ± 0.02)
Microgametocytes	
Infected erythrocyte	
Length	11.5–13.9 (12.3 ± 0.3)
Width	6.1–8.4 (7.3 ± 0.2)
Area	61.6–79.1 (69.4–24.4)
Infected erythrocyte nucleus	
Length	1.9–5.7 (5.0 ± 0.6)
Width	2.0–2.5 (2.3 ± 0.02)
Area	8.7–11.0 (9.8 ± 0.5)
Gametocyte	
Length	13.7–19.1 (17.1 ± 2.0)
Width	2.7–4.5 (3.7 ± 0.2)
Area	36.7–58.8 (47.9 ± 30.0)
Gametocyte nucleus	
Length	5.7–10.7 (8.6 ± 1.6)
Width	1.8–7.1 (3.0 ± 1.2)
Area	16.6–30.7 (23.6 ± 15.4)
Pigment granules	n/a
NDR	0.6–0.9 (0.7 ± 0.01)

n/a, no information is available as pigment granules were predominantly grouped (Fig. 2K-P) and difficult to count; NDR, Nuclear displacement ratio according to Bennett and Campbell (1972).

endothelial cells. Meront size, measured by the largest diameter, varied from 3 to 44 µm. In the heart, several meronts were located in cardiomyocytes. The meronts appeared solitary (Fig. 4M), in loose groups (Fig. 4N-P), or in juxtaposition with each other (Fig. 4-R-T). Single meronts varied from 8 to 35 µm in their largest diameter. Solitary meronts were oval (Fig. 4O, P) or elongated (Fig. 4M, N), while adjoining meronts showed various shapes such as round, oval, cubic, or angular forms (Fig. 4R, S). In the muscular layer of the gizzard, only a few meronts were detected, which were roundish (Fig. 4K) or elongated and reached about 40 µm in length. In the brain of one individual, only a single elongated meront was observed (Fig. 4L). Meronts found in the leg muscles looked similar to meronts found in the heart, being mainly elongated.

Most detected meronts were growing meronts, showing different stages of development, independent of their size. Few early meronts were identified and characterized by an amorphous appearance with prominent cytoplasmic clefts but lacked recognizable developing merozoites, indicating that meronts were still developing (Fig. 4D, E, K). The majority of meronts showed more advanced stages of development with developing merozoites arranged in irregular, sometimes angular-shaped cytomeres and separated by cytoplasmic clefts (Fig. 4C). In growing meronts, cytomeres seemed to gradually disappear while developing merozoites became more conspicuous by the aggregation of nuclei (Fig. 4F, I, J, Q-T). Beside growing meronts, a few nearly mature meronts were identified. In these, cytomeres or clefts were invisible or barely visible as they contained numerous discrete merozoites of about 0.5 µm (Fig. 4H, J, S). Occasionally, mature meronts located in larger blood vessels, probably representing detached infected host cells or liberated meronts. Meronts were commonly covered by a thin eosinophilic wall. The nuclei of infected cells were rarely recognizable. Bulb-like eosinophilic bulges were occasionally observed at the periphery of nearly mature meronts (Fig. 4F, H, I). These bulges were similar in colour and refractivity to the observed wall around the meronts, but their origin was unclear.

No inflammatory reactions were associated with the detected meronts.

Remarks: Dumbbell-like shape of growing gametocytes is a common feature in avian *Haemoproteus* spp. due to the presence of unfilled space between the gametocytes and the envelope of erythrocytes (Valkiūnas, 2005). However, this space remains and is readily visible in the majority of fully grown gametocytes only in *H. dumbbellus* n. sp. This is a unique character of *H. dumbbellus*, which can be readily distinguished from other species of haemoproteids parasitizing passeriform birds based on this feature.

Histological sections subjected to CISH revealed focal exo-erythrocytic signals in the brain of one bird, but the parasite was not found in the corresponding H & E-stained section. In other words, meronts certainly develop in brain, but their intensity is probably low, as in most organs with exception of the heart and lungs. In most CISH sections, exo-erythrocytic signals were deep purple and easy to distinguish (Fig. 4A-E, K-Q). However, in the meronts that grouped tightly in the heart, CISH signals were less intense, corresponding to more mature meronts with developed merozoites (Fig. 4R, T).

3.4. Haemoproteus hirundinis (lineage hDELURB2): gametocytes and exo-erythrocytic development

Gametocytes present in the blood films of *D. urbicum* displayed characteristics of *H. hirundinis* parasite, including the main diagnostic characters of the species (Fig. 1A-F), such as the pattern of growth around the host cell nucleus, without encircling it completely (Fig. 1D, F); absence of growing dumbbell-shaped gametocytes (Fig. 1C); fully grown gametocytes filling the poles of the erythrocytes and adhering to both the envelope of the host cell and its nucleus (Fig. 1D, F); strictly subterminal position of nuclei in fully grown macrogametocytes (Fig. 1D); and variable size of pigment granules (Fig. 1D, F). This parasite morphology was the same as described before (Valkiūnas, 2005) and its detailed description was not repeated here.

Exo-erythrocytic stages were found only in the pectoral muscles of two out of six infected *D. urbicum*. The megalomeronts were mostly elongated (Fig. 5I-X), following the muscle cells, while in transversal sections, they appeared oval (Fig. 5A-H). They were all covered by an eosinophilic, capsular-like wall of host origin. The megalomeronts varied in size and maturity; the largest were up to 353 µm at their longest diameter. Young megalomeronts were characterized by a more or less homogenous, light basophilic content without recognizable cytomeres (Fig. 5A-C). Growing megalomeronts showed small roundish cytomeres, from which merozoites seemed to bud off at the periphery, giving the structures a star-like shape (Fig. 5G, H, K). Mature megalomeronts were

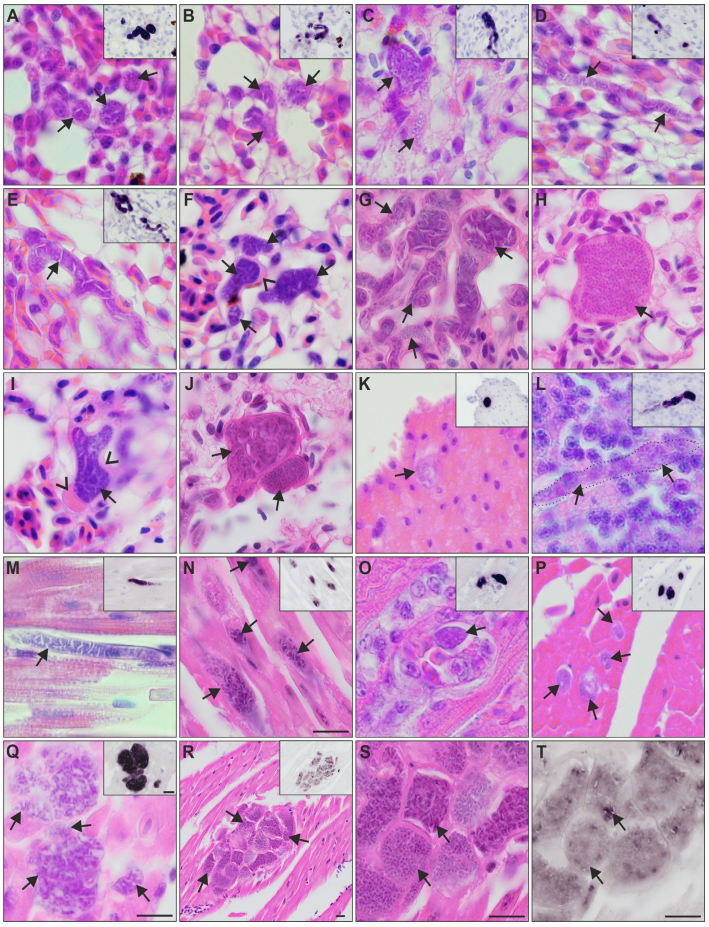

Fig. 4. Meronts of *Haemoproteus dumbbellus* (lineage hEMCIR01) found in (A-J) the lungs, (K) gizzard, (L) brain, and (M-T) heart of two yellow hammers *Emberiza citrinella*. The meront generic origin in H & E-stained preparations was confirmed by chromogenic *in situ* hybridization (CISH) using a *Haemoproteus* genus-specific probe indicated by a purple signal in the insets of panels A-E and K-R. Independently of their maturation stage, meronts (indicated by arrows) differed greatly in shape, being round (A, B, K), oval (O, P) or elongate (D, L-N), and ranging in size from less than 10 µm (P) to more than 50 µm (D, E, M, Q). Elongate meronts in the heart appear to follow the shape of the muscle cells (M, N), whereas meronts in the lung tissue (E) were often of capillary shape. Meronts were surrounded by a thin eosinophilic wall, occasionally with bulges of unclear origin located at the periphery of nearly mature parasites (F, I). Note the development of angular-shaped cytomeres separated by clefts, which is a characteristic feature of meront maturation in this parasite species. Such clefts are particularly well visible between cytomeres before merozoite formation (E, G, J-upper arrow), and they disappear in mature meronts, which are overfilled with discrete merozoites (H, J-lower arrow). R-T show the same group of meronts at different magnifications. Note the difference in maturation among meronts (R-T), with mature, roundish merozoites, characterized by weak CISH signals (S, T, lower arrow), or the still developing cytomeres and stronger CISH signals (S, T, upper arrow). Arrowheads indicate the eosinophilic wall. Scale-bar = 10 µm.

packed with numerous discrete merozoites, cytomeres were not identifiable (Fig. 5U-W).

Remarks: Growing megalomeronts were characterized by intense CISH signals (Fig. 5D, H), whereas in more advanced or mature megalomeronts, CISH signals appeared less intense (Fig. 5P-X). One megalomeront presented individual cytomeres with developing and mature merozoites (Fig. 5I-L), which is reflected by varying intensities of the CISH signals within the megalomeront. In one megalomeront, an inner envelope seemed to have detached from the capsular-like wall (Fig. 5Q-S), but still covered the parasites; it was not stained by the Haemo18S probe (Fig. 5T).

Moderate to severe inflammatory lesions were observed around most maturing megalomeronts (Fig. 5E-G, M-O, Q-S, U-W).

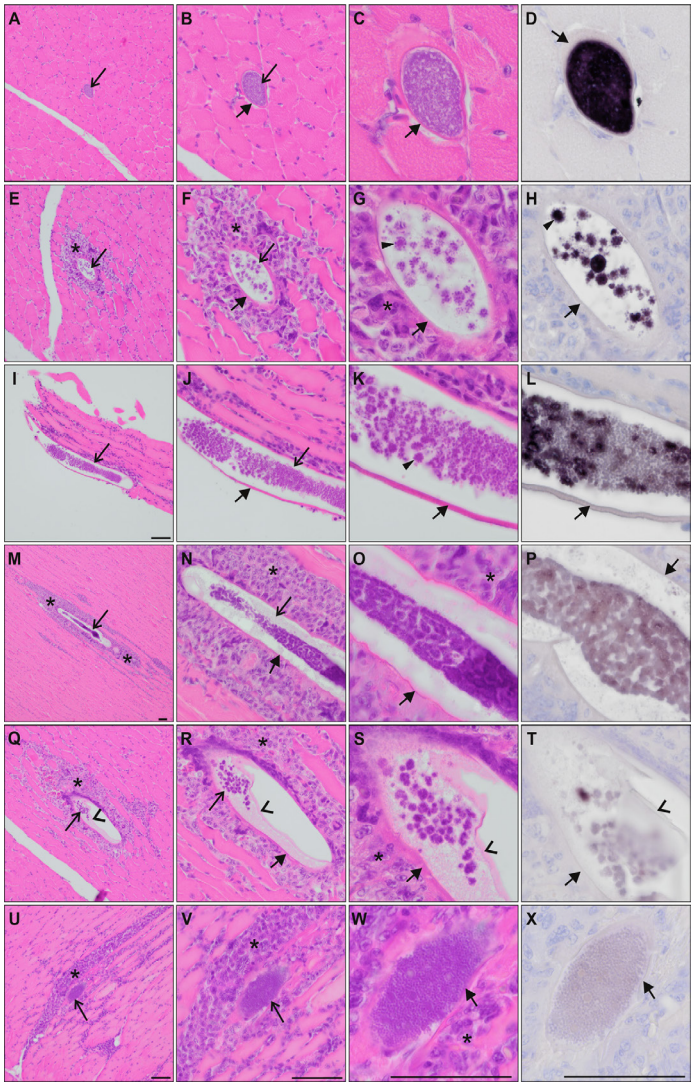

Fig. 5. Megalomeronts of *Haemoproteus hirundinis* (lineage hDELURB2) found in the (A-X) pectoral muscles of two common house martins *Delichon urbicum*. The megalomeront generic origin in H & E-stained preparations was confirmed by chromogenic *in situ* hybridization (CISH) using a *Haemoproteus* genus-specific probe (purple signal) on a subsequent section (D, H, L, P, T, X). Megalomeronts (indicated by the longer arrows) were elongate, following the muscles cells. In transverse sections, they appeared as roundish or oval bodies (A-D). Megalomeronts were covered by a capsular-like wall of host origin (indicated by the shorter arrows). Note the presence of predominantly small roundish cytomeres, with merozoites budding-off at their periphery, giving maturing cytomeres various star-like appearances (indicated by the triangles in G, H, K). Note the differences in probe signal intensity among megalomeronts of different maturation, varying from strong signal in young megalomeronts (D, H) over less intense signal in maturing megalomeronts (L) to almost no visible signal in fully mature megalomeronts (P, T, X). Inflammatory host cell infiltration (indicated by the asterisks) was observed around megalomeronts (E-G, I-K, M-O, Q-S, U-W). Note the presence of a thin membrane partially detached from the inner wall of the megalomeront (indicated by the arrowheads in Q-T), and still covering the parasite. Mature megalomeronts were overfilled with discrete, roundish merozoites (W). Scale-bar = 50 μm.

Neohapantotypes (gametocytes, *D. urbicum*, sampled in 2021.05.19 Ventė Cape, Lithuania, coll. Mikas Ilgūnas, accessions nos. 49428NS, 49436NS, 49437NS; exo-erythrocytic stages: pectoral muscles, accessions nos. 49433NS, 49434NS, 49440NS, 49441NS, other data as for gametocytes) were deposited to the NRC. Neoparahapantotypes (gametocytes, *D. urbicum*, 2021.05.19, Vente Cape, Lithuania, coll. Mikas Ilgūnas, accessions nos. 49429NS, 49438NS, 49439NS; exo-erythrocytic stages: pectoral muscles accessions nos. 49435NS, 49442NS, other data as for gametocytes) were deposited to the NRC. Neoparahapantotypes (gametocytes, *D. urbicum*, 2021.05.19, Ventė Cape, Lithuania, coll. Mikas Ilgūnas, accessions nos. G466253, G466254, G466258; exo-erythrocytic stages: accessions nos. G466255 – G466257, G46259 – G466261, other data as for gametocytes) were deposited to the Queensland Museum, Queensland, Australia. Additional voucher preparations from *D. urbicum* sampled at the type locality: gametocytes (accession nos. 49430NS – 49432NS, 49443NS – 49445NS) were deposited to the NRC.

4. Discussion

The key results of the present study are i) the discovery and description of exo-erythrocytic stages in two haemoproteid species and ii) the newly described *H. dumbbellus* n. sp. (lineage hEMCIR01) in *E. citrinella* and the assignment of the lineage hDELURB2 to the species *H. hirundinis*, a parasite of common swallows and house martins (Hirundinidae).

Haemoproteus dumbbellus n. sp. differs from other *Haemoproteus* parasites parasitizing passeriform birds by the presence of a readily visible space between the parasite and the erythrocyte envelope in fully grown gametocytes, giving the gametocytes a distinct dumbbell-like shape even at final stage of growth in the blood (Fig. 2H). This parasite is prevalent in *E. citrinella* (Passeriformes), its type vertebrate host.

Emberiza citrinella is a resident bird in Europe (Shirihai and Svensson, 2018a), with molecular records of the lineage hEMCIR01 from two other European countries besides Lithuania and Austria: 30 individuals were positive in Sweden (Ellis et al., 2020), and one in Slovakia (Šujanová et al., 2021). According to the MalAvi database, the lineage hEMCIR01 was also found in one *E. citrinella* from the United Kingdom (Dunn et al., 2014), however, the GenBank accession number indicated in the paper refers to a sequence (hEMRUT01), which differs from hEMCIR01 by one nucleotide. *Emberiza cirlus,* another bird species from the Emberizidae family, is a native resident in Southern Europe and Northern Africa (Shirihai and Svensson, 2018b), with records of hEMCIR01 in two individuals: one from Bulgaria (Dimitrov et al., 2010), and one from Morocco (Mata et al., 2015). The record of hEMCIR01 in *S. nebouxii* is the only one from South America, but the first 56 nucleotides are missing from the barcoding sequence, with the remaining nucleotides matching hEMCIR01 (Levin et al., 2011); it is thus uncertain whether the parasite really is hEMCIR01. As this record originated from a bird of a distant order and a different continent as all other records, it was likely the result of a contaminated sample (see Bensch et al., 2021). The record in *Phylloscopus trochilus* (Phylloscopidae), a long-distance migrant from Africa breeding in Europe and Russia (Shirihai and Svensson, 2018b), is from Sweden, where infected *E. citrinella* were also reported (Ellis et al., 2020). It is unknown if this record resulted from a competent host and complete development (gametocytes should be present) or an abortive infection, with PCR amplification from circulating sporozoite stages or exo-erythrocytic merozoites. Based on these limited molecular data available, *H. dumbbellus* appears to be of European distribution and probably is specific to its type vertebrate host and closely related *Emberiza* species.

In the present study, the lineage hDELURB2 was linked to *H. hirundinis*, confirming earlier predictions that the lineage might belong to this morphospecies (Chagas et al., 2019). Previously, *H. hirundinis* was only linked to the lineage hDELURB1 (Valkiūnas et al., 2014), which differs from hDELURB2 by 7 bp or 1.5% in the 478 bp *cytb* gene barcoding region. According to the MalAvi database, both lineages were frequently found in *D. urbicum* sampled in Europe, with 371 records for hDELURB1 (out of 1101 birds tested) and 271 records for hDELURB2 (out of 1082 birds tested) (MalAvi database, last access in September 2022 (Bensch et al., 2009)), indicating a similar prevalence for both lineages (25%) in this bird species. Apart from *D. urbicum*, hDELURB2 was also found in two barn swallows *Hirundo rustica* (Von Rönn et al., 2015, Garcia-Longoria et al., 2019), and 10 sand martins *Riparia riparia* (Ciloglu et al., 2020; Hahn et al., 2021), including one record under the lineage name hRIPRIP07, which is identical to hDELURB2 over the 478 bp section.

This study discovered the exo-erythrocytic stages of *H. dumbbellus* and *H. hirundinis*. These are the first known reports of tissue stages of haemoproteids, which were identified to species levels and parasitizing birds of the families Hirundinidae and Emberizidae. Both parasite species appeared phylogenetically closer to species developing meronts, e. g. *H. attenuatus* hROBIN1 (Hernández-Lara et al., 2021), *H. passeris* (Peirce, 1976), than to species developing only megalomeronts, e. g. *H. minutus, H. majoris, H. pastoris, H. syrnii* (Himmel et al., 2019; Ilgūnas et al., 2019, 2022; Ortiz-Catedral et al., 2019; Duc et al., 2020, 2021). However, exo-erythrocytic stages of *H. dumbbellus* and *H. hirundinis* were readily different from each other. *Haemoproteus dumbbellus* developed only meronts (Fig. 4), whereas *H. hirundinis* developed only megalomeronts (Fig. 5). The two species also showed different sites of exo-erythrocytic development: *H. hirundinis* megalomeronts were found only in the pectoral muscles, whereas meronts of *H. dumbbellus* were found in diverse organs, including lungs, liver, gizzard, heart and leg muscles (Table 1). This could indicate a preference in the parasite development, with *H. hirundinis* developing in specialized muscle cells, and *H. dumbbellus* in non-specialised cells, which are present in many organs. It is interesting to note that *H. passeris* was placed in the same clade as *H. dumbbellus* and other meront-forming species (Fig. 3) but has been reported to develop both meronts (Peirce, 1976) and megalomeronts (Burtikashvili, 1978; Valkiūnas and Iezhova, 2017) in house sparrows *Passer domesticus*, although this was found in different localities and studies (UK, Georgia). It remains unclear if these two authors were dealing with same or different genetic lineage of *H. passeris*.

Haemoproteus spp. with reported megalomeronts were scattered throughout the phylogeny (Fig. 3) and often developed in several organs: *H. pastoris* in the intestine, kidneys, lungs, oesophagus, gizzard, brain, spleen and trachea (Duc et al., 2021); *H. majoris* in the kidneys, lungs, liver, spleen, and intestine (Ilgūnas et al., 2019; Duc et al., 2020); *H. minutus* in the heart and gizzard (Ortiz-Catedral et al., 2019); *H. asymmetricus* in the heart (Himmel et al., 2019); *H. passeris* in the lungs and liver (Valkiūnas and Iezhova, 2017); *Haemoproteus sacharovi* in the gizzard (Farmer, 1964) and *Haemoproteus velans* and *H. syrnii* in the muscles (Groff et al., 2019; Ilgūnas et al., 2022). Interestingly, megalomeronts of *H. sacharovi, H. velans*, and *H. syrnii* all developed in muscle tissues (smooth muscles for *H. sacharovi*) and cluster in the phylogeny (Fig. 3). However, megalomeronts of *H. hirundinis*, also found exclusively in the pectoral muscles of its host, did not cluster with them. Megalomeront morphology is also quite different in all investigated species, with *H. hirundinis* being slender and of a smaller size compared with the megalomeronts found in the other three species (Farmer, 1964; Groff et al., 2019; Ilgūnas et al., 2022). Unfortunately, there is still no data available on the exo-erythrocytic development of species that do cluster more closely with *H. hirundinis* (Fig. 3), so any generalization about the

morphology and site of megalomeronts development remain premature based of available phylogenetic data.

For hDELURB1, a lineage previously attributed to *H. hirundinis*, data on exo-erythrocytic stages are absent. Due to the genetic similarity of the lineages hDELURB2 and hDELURB1, it is possible to presume that the latter would also develop megalomeronts in the pectoral muscles, analogous to a pattern observed in three *H. majoris* lineages, which all developed megalomeronts of similar morphology and location in different avian hosts (Ilgūnas et al., 2019; Duc et al., 2020). Further studies are needed to test this hypothesis.

Haemoproteus parasites were previously often neglected in veterinary medicine as thought to be relatively harmless to their avian hosts (Bennett et al., 1993). However, the recent reports of death of non-adapted birds due to molecularly proven haemoproteosis brought back interests on the study of the parasite exo-erythrocytic stages, which might markedly damage various organs (Ortiz-Catedral et al., 2019). A study recently reported cell necrosis associated with the development of *Haemoproteus* megalomeronts (Ilgūnas et al., 2022), while in the present study, inflammatory lesions were observed around the megalomeronts found in *D. urbicum*, but not around the small meronts found in *E. citrinella*. This could be due to their difference in size, inducing different host reactions or by the different stages of maturation of meronts/megalomeronts. When megalomeronts burst and release merozoites, the parasites are no longer protected by the capsular-like wall and become exposed to the hosts immune system, which can elicit an inflammatory response. The findings of this study demonstrate that natural hosts can be affected by haemoproteosis due to inflammatory reactions induced by exo-erythrocytic stages, which can be severe during excessive multiplication of the parasites. This calls for more attention to these pathogens and their veterinary importance not only in poultry and pet birds, but also in wildlife.

The markedly variable shape and small size of meronts makes them difficult to recognize in H & E-stained histological sections, as for example, in the case of the meront observed in the brain of *E. citrinella* (Fig. 4L). *Haemoproteus* meronts can sometimes appear outwardly similar to phanerozoites of some *Plasmodium* parasites (Valkiūnas, 2005). The CISH technique helps to locate such meronts and to confirm their generic origin using genus-specific probes, which target the *18S* ribosomal RNA of the parasites (Himmel et al., 2019). This method also provides insights into RNA expression of the parasites during the development of meronts and megalomeronts, as the intensity of the CISH signals should reflect the abundance of RNA molecules (Himmel et al., 2019). For example, the parasites should express more RNA as they actively multiply, and less RNA upon complete maturation leading to varying signal intensities among stages of different maturation. This pattern was well observed in megalomeronts of *H. hirundinis* (Fig. 5D, H, L, P, T, X), where young or growing megalomeronts showed deep dark CISH signals (Fig. 5D, H, L) and megalomeronts containing mature merozoites showed little CISH signals (Fig. 5X). One megalomeront also showed both darker (developing cytomeres) and less intense (mature merozoites) signals at the same time indicating asynchronous maturation of cytomeres and merozoites (Fig. 5L). A similar pattern was also observed in meronts of *H. dumbbellus* found in the heart muscle (Fig. 4T).

It is still unclear, whether a host will contain more exo-erythrocytic stages during high or low parasitaemia. In other words, it is unclear if high parasitemia is always accompanied with intensive exo-erythrocytic development. This information would be important for the decision process on whether, and at which time of infection to sacrifice infected birds aiming to investigate exo-erythrocytic development of the parasites. Among the four *E. citrinella* with 0.35% to 1.04% parasitaemia, the individual with the lowest infection intensity (0.35%) did not show any meronts in the sections analysed, while all the other individuals (0.69%, 0.98%, and 1.04% parasitaemia) exhibited meronts in diverse organs (Table 1). Among the six *D. urbicum* with 0.04% to 0.53% parasitaemia (Table 1), two individuals (0.34% and 0.53% parasitaemia) showed megalomeronts, but in the other two individuals with comparable parasitaemia (0.51% and 0.53%), the exo-erythrocytic stages were absent in the investigated sections (Table 1). A previous study on *H. pastoris* exo-erythrocytic development also reported the presence of megalomeronts during different parasitaemia (from 1% to 26%), except in one individual with a parasitaemia of 10% (Duc et al., 2021). Based on these data, no pattern emerges as to which parasitaemia intensity would be optimal for the investigation of exo-erythrocytic development in the avian host. Lower parasitaemia could imply ongoing exo-erythrocytic development with presence of mainly immature meronts and thus few infected erythrocytes yet, whereas higher parasitaemia could imply advanced exo-erythrocytic development with bursting meronts or being on the verge to burst (merozoites are released and infect erythrocytes). However, considering that meronts of different maturity were observed within the same individuals (Fig. 4) – a pattern also observed in megalomeronts of other species (Himmel et al., 2019; Ortiz-Catedral et al., 2019; Duc et al., 2021) and suggesting asynchronous merogony – the intensity of parasitaemia might not necessarily correlate with the abundance of merogonic stages in the tissues. In other words, the level of parasitaemia is likely a poor indicator of whether to find exo-erythrocytic stages in the organ of infected birds. Selection of birds with single infections is most important during fieldwork as it markedly simplifies further parasite species identification using general molecular primers and characterization of the developmental pattern of a single parasite.

The identification of haemosporidian species requires observation of gametocytes in blood films, and the detection of their exo-erythrocytic stages in tissues depends on histological work; both techniques require microscopic examinations. The specific parasite lineage is determined by molecular methods (PCR, DNA analysis). Due to the remarkable morphological diversity of tissue stages in haemosporidian parasites (Valkiūnas and Iezhova, 2017), the application of CISH is very helpful to prove generic identity of reported tissue stages. The combination of all these methods allows a more complete and accurate characterization of individual infections (Valkiūnas et al., 2006; Bensch and Hellgren, 2020). With about 177 species of *Haemoproteus* described (Valkiūnas and Iezhova, 2022), microscopic studies rely on experience and training to be able to identify the parasites (Valkiūnas, 2005; Valkiūnas and Iezhova, 2018, 2022); while molecular studies are relatively easy to apply and only require general to specific primers and PCR for amplification of the parasites' DNA and determination of lineages. Of about 1900 *Haemoproteus* lineages recorded so far, only 159 have been assigned to their corresponding morphospecies (MalAvi database, last access in September 2022, (Bensch et al., 2009)), leaving a large number of unlinked lineages and potentially new species. As most published studies do not investigate blood films, it is difficult to know whether a positive PCR amplification comes from the successfully developed parasites in their avian hosts (i.e. gametocytes developed), or from the injected sporozoites, which could not initiate infection or initiate only a partial development, resulting in the presence of incompletely developed exo-erythrocytic meronts and their remnants in the circulation (Valkiūnas and Iezhova, 2017). On the other hand, a negative molecular result could imply the absence of infection (no gametocytes, sporozoites or remnants of tissue stages), or result from mismatches between the primers and the parasite DNA. For example, we observed gametocytes in the blood films of six *D. urbicum*, but only two individuals were positive by PCR with the primers HAEMNFI/HAEMNR3 - HAEMF/

HAEMR2 (Bensch et al., 2000; Hellgren et al., 2004). However, when using the primers PLAS1F/HAEMNR3 and 3760F/HAEMJR4 (Beadell et al., 2004; Hellgren et al., 2004; Duval et al., 2007; Pérez-Rodríguez et al., 2013), all samples were positive. Comparing the primer sequences HAEMF/HAEMR2 to a longer sequence of hDELURB2 previously submitted (MK843311 (Chagas et al., 2019)), three mismatches were found for both the forward and reverse primers, which could explain lower primer affinity and amplification rate. Thus, alternative primers are recommended for screening of *D. urbicum* or bird species infected with closely related parasite species. Ultimately, both molecular and microscopic methods are crucial for gaining a more complete picture of the lineage diversity present in a given bird species and for the discovery of new lineages and parasite species.

In summary, this study contributes to the knowledge of exo-erythrocytic development of avian Haemoproteidae species due to the discovery of the tissue stage in two *Haemoproteus* parasites – *H. dumbbellus* n. sp. and *H. hirundinis*. The new species, *H. dumbbellus*, and its exo-erythrocytic stages were described from *E. citrinella* infected with the lineage hEMCIR01; only meronts of markedly different shapes were observed in the lungs, heart, brain, liver, leg muscles and gizzard. The lineage hDELURB2 was attributed to *H. hirundinis* observed in *D. urbicum*, and its exo-erythrocytic stages – megalomeronts – were only found in the pectoral muscles. The megalomeronts were of unique morphology among avian haemoproteids due to the star-like appearance of developing cytomeres. This study highlights the remarkable diversity of exo-erythrocytic stages throughout *Haemoproteus* spp., which develop meronts or megalomeronts and infect one or several organs. Analysis of the available data suggest that the exclusive development of meronts might be restricted to fewer *Haemoproteus* species than previously thought, while megalomeronts seem to develop in more parasite species and probably is the predominate stage during exo-erythrocytic development of avian haemoproteids. Future investigations of other common *Haemoproteus* species are needed to examine which exo-erythrocytic stage (meront, megalomeront, or both) in which parasite species might predominantly occur, and whether molecular phylogenies can be used in practical parasitological work to predict patterns of exo-erythrocytic development using simply DNA sequence information.

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PAPER V

Comparative analysis of the exo-erythrocytic development of five lineages of *Haemoproteus majoris*, a common haemosporidian parasite of European passeriform birds.

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Comparative Analysis of the Exo-Erythrocytic Development of Five Lineages of *Haemoproteus majoris*, a Common Haemosporidian Parasite of European Passeriform Birds

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Abstract: *Haemoproteus* parasites (Apicomplexa, Haemosporida) are widespread pathogens of birds, with a rich genetic (about 1900 lineages) and morphospecies (178 species) diversity. Nonetheless, their life cycles are poorly understood. The exo-erythrocytic stages of three *Haemoproteus majoris* (widespread generalist parasite) lineages have been previously reported, each in a different bird species. We aimed to further study and compare the development of five *H. majoris* lineages—hCCF5, hCWT4, hPARUS1, hPHSIB1, and hWW2—in a wider selection of natural avian hosts. A total of 42 individuals belonging to 14 bird species were sampled. Morphospecies and parasitemia were determined by microscopy of blood films, lineages by DNA-barcoding a 478 bp section of the cytochrome *b* gene, and exo-erythrocytic stages by histology and chromogenic in situ hybridization. The lineage hCWT4 was morphologically characterized as *H. majoris* for the first time. All lineage infections exclusively featured megalomeronts. The exo-erythrocytic stages found in all examined bird species were similar, particularly for the lineages hCCF5, hPARUS1, and hPHSIB1. Megalomeronts of the lineages hWW2 and hCWT4 were more similar to each other than to the former three lineages. The kidneys and gizzard were most often affected, followed by lungs and intestines; the site of development showed variation depending on the lineage.

Keywords: *Haemoproteus*; birds; megalomeronts; cytochrome *b*; histology; chromogenic in situ hybridization

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1. Introduction

Haemoproteus species (Apicomplexa, Haemosporida, Haemoproteidae) are parasites of birds and found on all continents except Antarctica [1,2]. These pathogens multiply asexually and produce gametocytes in their avian hosts before being transmitted to their dipteran vectors (biting midges, Ceratopogonidae), in which the fusion of the gametes and the development of sporozoites occur [1,3].

Anemia is an acknowledged symptom caused by *Haemoproteus* parasites, with the gametocytes developing in the erythrocytes [4,5]. However, the exo-erythrocytic development, which occurs in different organs, was initially assessed to be less harmful for their avian hosts compared with that of haemosporidian parasites of the genera *Plasmodium* and *Leucocytozoon* [6], and research on *Haemoproteus* tissue stages remained scarce. Recent studies reported exo-erythrocytic stages of several *Haemoproteus* species [7–15], not only increasing and changing traditional knowledge of these parasites but also pointing out how little is still known about their development in avian hosts.

Most reports of exo-erythrocytic stages are either from hosts found naturally dead [7,8,10,11,15] or from individuals that have been purposely targeted for their parasite infection

and euthanized [9,12–14]. In several cases, no exo-erythrocytic stages of *Haemoproteus* spp. were found in histological slides of birds confirmed positive by blood film microscopy and/or DNA barcoding, despite extensive searches [11,13–15].

Haemosporidian parasites are investigated and characterized genetically by the amplification and sequencing of a 478 bp DNA-barcode region of the mitochondrial cytochrome *b* (*cyt b*) gene [16]. Morphospecies are usually described using the morphological characteristics of gametocytes and their host cells in blood films from infected birds [1,17,18]. However, not all described species have their molecular characterization, and not all lineages are linked to a species yet. In some cases, several lineages can be linked to the same morphospecies, e.g., *H. majoris* [9,19] and *H. tartakovskyi* [20] (MalAvi database, Lund University, Lund, Sweden, http://130.235.244.92/Malavi/ (accessed on 25 June 2023). [16]).

Haemoproteus majoris is a species of interest among the *Haemoproteus* parasites, with several lineages (hCCF5, hPARUS1, hPHSIB1, hPHYBOR04, and hWW2) (MalAvi database [16]; [9,19]) reported in 54 species of birds in Asia, Europe, Africa, and North America (MalAvi database [16]). A sixth lineage, hCWT4, has been associated with this species [21], but it has never been formally described morphologically as *H. majoris*. This species has been studied for its specificity (it is a generalist parasite [21]), its vectors (*Culicoides impunctatus* is a competent vector for the lineages hPARUS1 and hPHYBOR04 [9,22]), and its exo-erythrocytic development (parasites of the lineages hPARUS1, hPHYBOR04 [9], and hPHSIB1 [12] developed into megalomeronts). The exo-erythrocytic stages of *H. majoris* were reported in three different bird species (*Parus majoris*, *Turdus pilaris* [9], and *Phylloscopus sibilatrix* [12]), and all found tissue stages were megalomeronts of similar morphologies, which were predominantly seen in the kidneys. However, it is unknown whether all *H. majoris* lineages develop similarly in different avian hosts. Further comparative studies are needed to answer this question, which is an important issue in understanding the mechanisms of exo-erythrocytic development during avian haemoproteosis. Due to the large diversity of lineages and the occurrence in a large variety of different bird hosts, *H. majoris* is an excellent model organism for such a comparative study.

We aimed to clarify the identity of hCWT4 as *H. majoris* and to investigate and provide a comparative analysis of the exo-erythrocytic stages of different *H. majoris* lineages (hCCF5, hCWT4, hPARUS1, hPHSIB1, and hWW2) in different host species and in different seasons. Patterns of development within this parasite species were investigated in regard to the exo-erythrocytic stages' morphology, distribution, and localization.

2. Materials and Methods

2.1. Sampling

Birds caught at the Ornithological Station, Ventes Ragas, Lithuania (55°20′38.93″ N, 21°11′34.05″ E), were sampled and investigated for haemosporidian parasites beginning in May 2016 to 2019, 2021, and 2022 (spring) and in September 2020 (autumn). Birds were additionally caught using mist nests in the Labanoras Forest, Lithuania (55°12′25.77″ N, 25°56′26.47″ E), in June 2018 and 2019 (spring) and July 2020 (summer). Blood was collected by puncturing the branchial vein of the bird. A drop of blood was immediately used to prepare at least three blood films, which were air-dried and fixed with absolute methanol [1]. Blood collected in a heparinized capillary was transferred into a SET buffer (0.05 M Tris, 0.15 M NaCl, 0.5 M EDTA, pH = 8.0) [23] tube for molecular analysis and stored at +4 °C in the field and at −20 °C in the laboratory.

One blood film per individual bird was immediately stained for 15 min with a 30% Giemsa solution and examined for approximately 10 min with an Olympus CX26 microscope (Olympus, Tokyo, Japan) to determine potential infections in captured birds in the field. This procedure enabled the selection of targeted infected birds and the release of all other individuals at the study sites. The remaining blood films were stained with a 10% Giemsa solution for 1 h. Birds infected with *H. majoris*, as observed by microscopic examination, were euthanized, and internal organs were collected; brain, heart, lungs, liver, kidneys, spleen, and skeletal muscles were collected from birds of all years, and trachea, esophagus, gizzard, intestine, pancreas, and reproductive organs were additionally

collected from birds collected in 2019 to 2022. The organs were fixed in 10% formalin and later embedded in paraffin wax.

In July 2021, one black redstart (*Phoenicurus ochruros*) found dead in Lithuania (54°52′58.4″ N 25°25′38.6″ E) with a skull fracture was frozen and brought to the P. B. Šivickis Laboratory of Parasitology, Nature Research Centre, Lithuania. In October 2021, one long-tailed tit (*Aegithalos caudatus*) found dead at the Ventes Ragas Ornithological Station, was frozen until dissection. Both birds were dissected, the heart was pressed to a glass slide to prepare a blood film, little pieces of organs were collected in 96% EtOH and stored at +4 °C, and the organs were fixed in formalin for 24 h, washed in distilled water for 1 h, transferred to 70% EtOH, and processed as the other samples.

2.2. Blood Film Microscopy

Haemosporidian parasite species were determined by screening the blood films with an Olympus BX61 microscope (Olympus, Tokyo, Japan) at ×1000 magnification [1]. Parasitemia intensity was calculated as the number of infected erythrocytes per 1000 erythrocytes or per 10,000 erythrocytes in case of low infection and expressed as percentage [24].

Images of the parasites were taken using the same microscope equipped with an Olympus DP70 digital camera and AnalySIS FIVE software (Olympus, Tokyo, Japan). The software Adobe InDesign CS6 (Adobe, https://www.adobe.com/products/indesign.html (accessed on 25 June 2023)) was used to prepare the gametocytes figure.

2.3. Histological Analysis

Histological sections of 2–3 µm were cut with a microtome, mounted on glass slides, stained using haematoxylin and eosin (H&E), and investigated for exo-erythrocytic stages using ×100, ×200, and ×400 magnifications with an Olympus BX41 or BX51 microscope (Olympus, Japan).

Chromogenic in situ hybridization (CISH) was applied on additional sections, at least one per individual, following the original protocol [8,25]. A genus-specific oligonucleotide probe (Haemo18*S*), which targets the *18S* ribosomal RNA of *Haemoproteus* parasites, was used to confirm the generic origin of the observed tissue stages [8].

Pictures of the tissue stages were taken using the camera DP12 with the software DP-SOFT or the camera UC90 with the software cellSens (Olympus, Tokyo, Japan), respectively. The software CorelDraw 2019 (RRID:SCR_014235, https://www.coreldraw.com/en/) was used to prepare the figures. (accessed on 9 November 2022)

For all bird individuals single-infected with *H. majoris* hPARUS1, the number of sections investigated and the number of sections positive for exo-erythrocytic stages were counted and reported. The number of very young megalomeronts and other megalomeronts (referring to growing, mature, and ruptured megalomeronts) were also counted and reported. It is to be noted that very young megalomeronts can be found on up to three sequential sections of organs, but most often, one very young megalomeront was observed in only one section. Other, bigger megalomeronts are most often observed on several sequential sections. However, not all sections investigated were sequential. Therefore, the detection of megalomeronts is biased greatly for big megalomeronts compared with very young megalomeronts. Due to the fact that the actual shape (oval, round, long, or slender) of megalomeronts is difficult to predict, the choice was made to not try to distinguish the real number of the bigger megalomeronts.

2.4. Molecular Analysis

DNA extractions were done using the SET-buffer-stored blood following the ammonium acetate protocol [26] or the DNeasy Blood & Tissue Kit (QIAGEN, Venlo, The Netherlands). The extracted DNA was used as template in a nested PCR following the original protocols [23,27] with the outer primer pair HAEMNFI/HAEMNR3 and inner primer pairs HAEMF/HAEMR2 for detection of *Haemoproteus* and *Plasmodium* parasites and HAEMFL/HAEMR2L for *Leucocytozoon* parasites. A negative control (ddH_2O) and a

positive control (sample known to be infected with *Plasmodium* and *Leucocytozoon* parasites) were used in all PCRs to check for possible contamination and efficiency of the PCR. PCR products were run on 2% agarose gel. Positive PCR products were sequenced in both directions with the Big Dye Terminator V3.1 Cycle Sequencing Kit and the ABI PRISM^{TM} 3100 capillary sequencing robot (Applied Biosystems, Foster City, California) or sent for sequencing to Microsynth (Microsynth, Austria). One sample was subjected to cloning of the *18S* ribosomal RNA of parasites following the protocol of a previous study [28].

Chromatograms were checked for possible mixed infections (more than one peak for a base) using the software Geneious Prime 2022.0.2 (https://www.geneious.com, last accessed on 9 November 2022) or Bioedit (https://bioedit.software.informer.com last accessed on 9 November 2022). Sequences were subjected to BLAST searches in MalAvi database [16] and NCBI GenBank database (National Library of Medicine, Bethesda, Maryland, https://www.ncbi.nlm.nih.gov/genbank/, last accessed on 9 November 2022) to determine the lineages. Sequences obtained from infections with no more than two lineages from the same genus were deposited in GenBank OR143042–OR143096 (i.e., sequences from the two bird individuals with mixed infections of possibly four lineages of *H. majoris* were not deposited).

Genetic pairwise distances between the lineages were calculated in the software MEGA-X: Molecular Evolutionary Genetics Analysis across computing platforms [29].

2.5. Deposition of Voucher Preparations

Vouchers of blood preparations (accessions 49493 NS–49495 NS, 49531 NS, 49567 NS, 49568 NS, 49595 NS, 49615 NS–49617 NS) and histological preparations (accessions 49496 NS–49530 NS, 49532 NS–49566 NS, 49569 NS–49594 NS, 49596 NS–49614 NS, 49618 NS–49632 NS, 49642 NS and49643 NS) were deposited to the Nature Research Centre, Vilnius, Lithuania. Vouchers of blood and histological preparations (accessions G466275-G466294) were deposited at the Queensland Museum, Brisbane, Australia.

3. Results

3.1. Species and Lineage Identification

In total, 42 bird individuals with *H. majoris* infections were dissected (Tables 1 and S1): 1 *Aegithalos caudatus*, 6 *Fringilla coelebs*, 7 *Cyanistes caeruleus*, 1 *Parus cristatus*, 8 *Parus major*, 1 *Parus montanus*, 2 *Phylloscopus sibilatrix*, 1 *Phylloscopus trochilus*, 1 *Phoenicurus ochruros*, 1 *Phoenicurus phoenicurus*, 3 *Sylvia atricapilla*, 4 *Sylvia communis*, 3 *Sylvia curruca*, and 3 *Sylvia nisoria*. All infections were confirmed by microscopic examination of the blood films with *H. majoris* characteristics (Figure 1), except three individuals (*Aegithalos caudatus*, *Phylloscopus sibilatrix*, and *Phoenicurus phoenicurus*) whose blood films were negative or not usable (from dead birds). One *H. majoris* infection was found in co-infection with *Leucocytozoon* sp. and eleven with other *Haemoproteus* spp., as confirmed by microscopy (Tables 1 and S1). Parasitemia intensity ranged from 0.01% to 5.1% in single infections of *H. majoris* (Tables 1 and S1).

The gametocytes present in the hCWT4 infection display the main *H. majoris* characteristics (Figure 1E–H): gametocytes grow around the erythrocyte nuclei but do not encircle them; dumbbell-shaped growing gametocytes are present; and pigment granules are roundish, sometimes oval, of medium to small size, and are randomly scattered. The parasite morphology was the same as described before, and its description is not repeated here [1].

Molecular analysis confirmed the *H. majoris* infections of the dissected birds and revealed five co-infections of different *H. majoris* lineages, two co-infections with other *Haemoproteus* lineages, and eleven co-infections with *Leucocytozoon* lineages (Tables 1 and S1). In total, seven birds were found infected only with the hCCF5 lineage, eight with hCWT4, twelve with hPARUS1, three with hPHSIB1, and seven with hWW2; these numbers exclude the samples with mixed infection of different *H. majoris* lineages (Tables 1 and S1). Nine of the identified co-infections by microscopy were not picked up by PCR, and only one lineage was identified in those infections.

Haemoproteus species were identified by microscopic examination in samples with co-infections where exo-erythrocytic stages were found (Table 1).

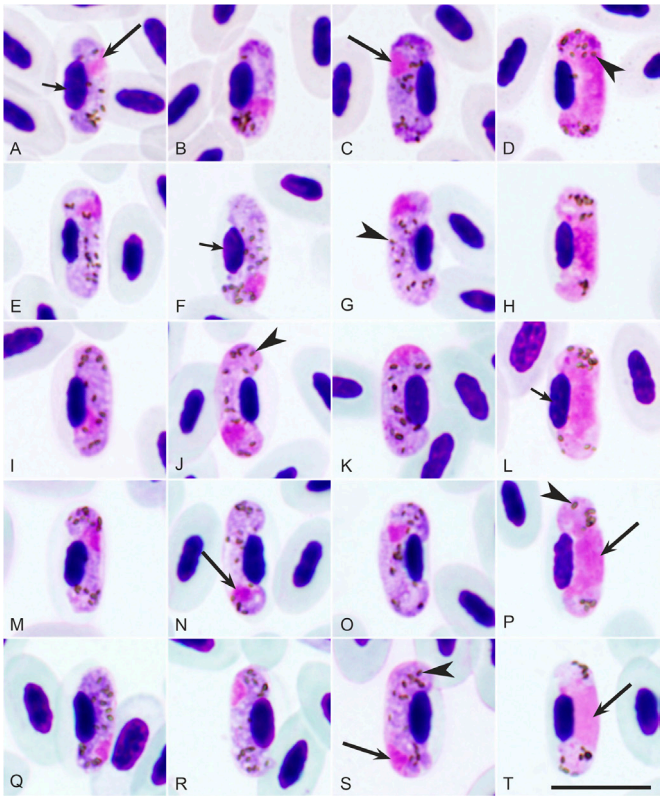

Figure 1. Gametocytes of different *Haemoproteus majoris* lineages found in blood films: hCCF5 (**A**–**D**) in *Fringilla coelebs*, hCWT4 (**E**–**H**) in *Sylvia curruca*, hPARUS1 (**I**–**L**) in *Cyanistes caeruleus*, hPHSIB1 (**M**–**P**) in *Phoenicurus ochruros*, and hWW2 (**Q**–**T**) in *Phylloscopus trochilus*. Note that gametocytes of all lineages are morphologically indistinguishable; the fully grown gametocytes (**C**,**D**,**G**,**H**,**K**,**L**,**O**,**P**,**S**,**T**) of all lineages reach the poles of erythrocytes but do not completely encircle the erythrocyte nuclei, which were displaced laterally. Pigment granules were similar in size, form, and number in gametocytes of all lineages. Long arrow—gametocyte nucleus; short arrow—erythrocyte nucleus; arrowhead—pigment granules. Scale bar: 10 μm.

Table 1. Data on individuals infected with *Haemoproteus majoris* showing exo-erythrocytic stages. Information is organized by lineage of infection and data are provided for the season of collection, parasitemia, host species, parasite identification (through microscopy and PCR), and organs in which the exo-erythrocytic stages were found both in haematoxylin and eosin (H&E)-stained sections and the sections treated with chromogenic in situ hybridization (CISH) with the probe Haemo18S.

Identity No. [#]	Parasite -Mia (%)	Host Species	Parasite Species and Cytochrome *b* Lineages	Tissue Stages Seen in H&E and CISH Sections [‡]								
				He	Lu	Li	Sp	Ki	Br	Mu	Int	Gi
1 Sp	0.7 [a]	*Fringilla coelebs*	*H. majoris* (hCCF5), *Haemoproteus fringillae* [b], *Leucocytozoon* sp. (lBRAM3) [c]	+	H	-	-	-	-	-	na	na
3 Sp	2.8 [a]	*F. coelebs*	*H. majoris* (hCCF5), *H. fringillae* [b], *Haemoproteus magnus* [b], *Haemoproteus* sp. [b]	-	+/H	-	-	-	-	-	na	na
19 Sp	0.2	*F. coelebs*	*H. majoris* (hCCF5)	-	-	-	-	-	-	-	-	+ [e]
27 Sp	0.2 [a]	*F. coelebs*	*H. majoris* (hCCF5), *Mixed infection* [b]	-	+	-	-	+	-	-	+	+
28 Sp	0.1 [a]	*F. coelebs*	*H. majoris* (hCCF5), *L.* sp. (lEMCIR02) [c], *Mixed infection* [b]	-	-	-	-	-	-	-	+ [f]	+
3 Su	0.02	*Parus cristatus*	*H. majoris* (hCCF5)	-	-	-	-	-	-	-	+ [e,f]	-
6 Sp	1.3	*Sylvia communis*	*H. majoris* (hCWT4)	-	-	-	-	-	-	+ [d]	na	na
18 Sp	1.6	*Sylvia curruca*	*H. majoris* (hCWT4)	-	-	-	-	-	-	-	+	+
21 Sp	1.1	*S. curruca*	*H. majoris* (hCWT4)	-	-	-	-	-	-	-	-	+ [e]
22 Sp	1	*S. curruca*	*H. majoris* (hCWT4)	-	-	-	-	-	-	-	-	+
7 Sp	1.2	*Cyanistes caeruleus*	*H. majoris* (hPARUS1)	+	+	+	-	+	-	-	na	na
23 Sp	5.1	*C. caeruleus*	*H. majoris* (hPARUS1), *L.* sp. (lPARUS14) [c]	-	+	+	-	+	-	-	-	+
2 Au	0.1	*C. caeruleus*	*H. majoris* (hPARUS1)	-	-	-	-	+	-	-	-	-
4 Au	0.1	*C. caeruleus*	*H. majoris* (hPARUS1), *L.* sp. (PARUS4) [c]	-	-	-	-	+ [e]	-	-	-	-
2 Su	0.5	*Parus major*	*H. majoris* (hPARUS1)	-	+	-	-	+	-	-	+ [e,f]	-
6 Au	0.07	*P. major*	*H. majoris* (hPARUS1)	-	-	-	+ [e]	+	-	-	-	-
7 Au	0.01	*P. major*	*H. majoris* (hPARUS1), *L.* sp. (lPARUS18) [c]	-	-	-	-	+ [e]	-	-	-	-
8 Au	0.03	*Parus montanus*	*H. majoris* (hPARUS1)	-	-	-	-	+	-	-	-	-
9 Sp	NEG	*Phylloscopus sibilatrix*	*H. majoris* (hPHSIB1)	-	-	-	-	+	-	-	na	na
4 Sp	0.6	*P. major*	*Haemoproteus majoris* (hWW2/hCWT4/hPARUS1/hPHSIB1)	-	-	+	-	-	-	-	na	na
1 Su	0.1	*P. major*	*H. majoris* (hWW2/hCWT4/hPARUS1/hPHSIB1)	-	-	-	-	-	+ [d]	-	-	-
12 Sp	6.3 [a]	*Sylvia atricapilla*	*H. majoris* (hWW2), *Haemoproteus parabelopolskyi* [b], *L.* sp. (lSYAT22) [c], *Leucocytozoon majoris* [b]	+	+	-	-	-	-	+	na	na
24 Sp	4.7 [a]	*S. atricapilla*	*H. majoris* (hWW2), *H.* sp.1 [b], *H. parabelopolskyi* [b], *H.* sp 2. [b], *L.* sp. (lCOCOE09) [c]	+	-	-	-	+	-	-	-	+
17 Sp	0.7	*Phylloscopus sibilatrix*	*H. majoris* (hWW2 and hPHSIB1)	-	-	-	na	+	-	-	+ [e]	-
29 Sp	0.6	*Phylloscopus trochilus*	*H. majoris* (hWW2)	-	-	-	-	-	-	-	-	+
20 Sp	1.1	*Phoenicurus ochruros*	*H. majoris* (hPHSIB1 and hWW2 [g])	+	-	-	-	-	+	-	+	+

[#] "Sp" spring; "Su" summer; "Au" autumn. [a] Overall parasitemia intensity for all parasites present (no distinction between the species present in co-infections). [b] Parasite only seen during microscopy but not detected by PCR; [c] parasite not observed during microscopic examination of blood films, so only PCR results are available. [‡] The trachea, esophagus, testes, or ovaries were negative for all individuals and excluded from this table; He: heart; Lu: lungs; Li: liver; Sp: spleen; Ki: kidneys; Br: brain; Mu: skeletal muscle; Int: intestine; Gi: gizzard; "-" samples negative for *Haemoproteus* exo-erythrocytic stages in both H&E and CISH sections; "+" samples positive for *H. majoris* exo-erythrocytic stages in both H&E and CISH sections; "na" organ not collected, or the sections are not present for the analysis of tissue stages; "H" samples positive for exo-erythrocytic stages of other *Haemoproteus* spp. in both H&E and CISH sections; [d] parasite was not present in the consecutive histological section, so not tested by CISH; [e] stage present only in CISH; [f] stages found in the pancreas, not the intestine. [g] Lineage obtained by cloning [28].

Birds negative for exo-erythrocytic stages were excluded from this table and are present in Table S1.

H. majoris lineages differed from 0.2 to 1.3% (1 to 6 nucleotides) in the 478 bp *cyt b* barcode region (Table 2).

Table 2. Pairwise distances in percentage between the different *Haemoproteus majoris* lineages in the 478 bp cytochrome *b* sequence.

Lineage	hCWT4	hPARUS1	hPHSIB1	hPHYBOR04	hWW2
hCCF5	0.6	0.6	1.3	0.8	0.8
hCWT4		0.4	0.6	0.2	0.2
hPARUS1			1.0	0.6	0.6
hPHSIB1				0.8	0.4
hPHSYBOR04					0.4

3.2. Haemoproteus majoris Exo-Erythrocytic Stages

Only megalomeronts were found during all single *H. majoris* infections. Meronts were only present in samples with mixed infections of *H. majoris* and other *Haemoproteus* species. Megalomeronts were found in 25 of the 42 investigated birds. However, not all organs were collected from 10 of the 17 individuals that were negative for exo-erythrocytic stages (Tables 1 and S1).

3.2.1. Exo-Erythrocytic Stages Morphology

hPARUS1, hCCF5, and hPHSIB1 lineages of *H. majoris* displayed similar morphology to that of megalomeronts (Figures 2A–D,J–S and S1, S3), which were surrounded by a thick capsular-like wall and possessed variously shaped interconnected cytomeres in which merozoites developed. Some megalomeronts in hCWT4 and hWW2 infections were slightly different in their morphology (Figure 2E–I,T–X). Mainly, the cytomeres were present, but they were denser and interconnected more tightly (compare Figure 2F,G,W,X with Figure 2A–C,J). Interestingly, their host species also differed, with hPARUS1 found predominantly in *Parus* spp., hCCF5 in *Fringilla coelebs*, and hCWT4 and hWW2 in *Sylvia* spp. (Tables 1 and S1).

Some of the megalomeronts found in the gizzard with hCWT4 infection (Figure 2E) developed in the koilin layer and looked degenerated, reacting poorly with the Haemo18S probe, while other megalomeronts at a similar stage of growth showed a deep purple CISH signal. Other parasite tissue structures were found in the gizzard with varying shapes, some of them appearing ruptured with very poor to no CISH signal (Figures S2 and S4).

Megalomeront morphology of the same *H. majoris* lineages' infection did not differ between host species. For example, megalomeronts of hPARUS1 infections found in *Cyanistes caeruleus*, *Parus major*, and *Parus montanus* were of the same morphology (Figures 2J–N and S3).

Very young megalomeronts (less than 20 µm in diameter) were found in 10 birds, 1 in hCCF5, 1 in hCWT4, 5 in hPARUS1, 1 in hPHSIB1 lineage infections, and 2 in *H. majoris* co-infections. The host cell nucleus was present and slightly enlarged (Figures 2D,I,N,S, 3B–D and S3). The host cell nucleus was not seen in more advanced, developing, and mature megalomeronts (Figures 2A–C,E–H,J–M,O–R,T–W and S1–S5).

Inflammatory reactions were observed around some megalomeronts. The inflammatory infiltrates were predominantly lymphohistiocytic, and only occasionally were single heterophils seen. Inflammation was present around seemingly intact megalomeronts, where the intensity could vary between single inflammatory cells (Figure 2B) and several layers of them (Figure 2O–R,T). Adjacent to damaged or ruptured parasitic structures the inflammation was generally severe.

Individuals infected with the same parasite lineage and a similar intensity of parasitemia often featured megalomeronts (Table 1) but not always (Table S1).

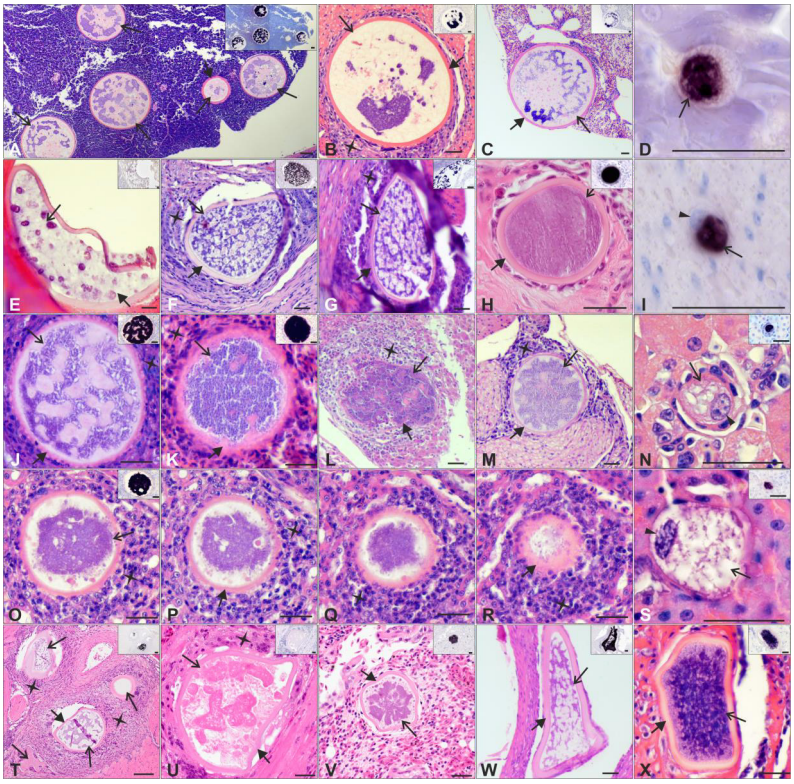

Figure 2. Megalomeronts of different *Haemoproteus majoris* lineages: hCCF5 (**A**–**D**), hCWT4 (**E**–**I**), hPARUS1 (**J**–**N**), hPHSIB1 (**O**–**S**), and hWW2 (**T**–**X**) in haematoxylin and eosin (H&E)-stained sections and their corresponding images after chromogenic in situ hybridization (CISH) treatment (inserts and (**D**,**I**). Megalomeronts were found in pancreas (**A**,**D**), gizzard (**B**,**E**–**I**,**M**,**W**,**X**), lungs (**C**,**K**,**V**), kidneys (**J**,**N**–**S**), liver (**L**), muscles (**T**), and heart (**U**) of their host. Note the variously shaped, interconnected cytomeres in developing megalomeronts (**A**–**C**,**J**,**M**) and the more densely aggregated and connected cytomeres in megalomeronts of hCWT4 and hWW2 (**F**,**G**,**T**,**W**,**X**). Very young megalomeronts (**D**,**I**,**N**,**S**) were found in the infections of four lineages. The host cell nucleus was slightly enlarged and visible in the very young megalomeronts (**D**,**I**,**N**,**S**) but absent in more developed (**A**–**C**,**E**–**H**,**J**,**K**,**M**,**O**–**P**,**T**–**X**) and ruptured (**L**) megalomeronts. Ruptured megalomeronts (**L**) were found in the liver of one individual. One megalomeront was found to appear in serial sections (**O**–**R**) showing how different its morphology and size can be depending on the analyzed section. Megalomeronts were found solitary in the tissues, and sometimes several megalomeronts were found in the same section located close to each other (**A**,**T**). Inflammatory reactions were observed around several megalomeronts (**B**,**F**,**G**,**J**–**M**,**O**–**R**,**T**,**U**). Megalomeronts were surrounded by a thick capsular-like wall, except for the very young ones. Cytomeres were readily visible in stages of advanced development. Long arrow: megalomeront; Short arrow: capsular-like wall; Cross: inflammatory reaction; Arrowhead: enlarged host cell nucleus. Scale bar: 100 µm (**A**,**T**); 25 µm (**B**–**S**,**U**–**X**).

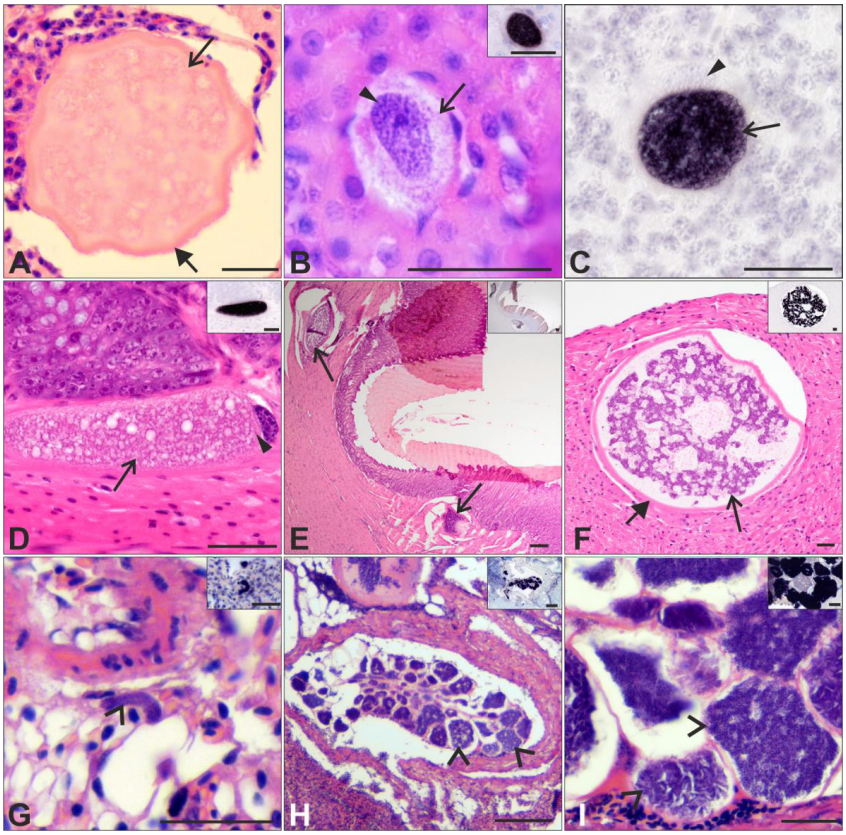

Figure 3. Exo-erythrocytic stages (megalomeronts (**A**–**F**), and meronts (**G**–**I**)) of different *Haemoproteus* species in co-infection with *H. majoris* found in H&E-stained sections and CISH-tested sections (inserts and (**C**)): co-infections of different *H. majoris* lineages—hWW2, hPHSIB1, hPARUS1, and hCWT4—present (**A**) and hWW2 with hPHSIB1 (**B**–**F**) present; *H. majoris* hCCF5 with *H. fringillae* (unknown lineage) (**G**); *H. majoris* hCCF5 with *H. fringillae* and *H. magnus* (unknown lineages) (**H**,**I**). The tissue stages were found in the brain of a *Parus major* (**A**) and a *Phoenicurus ochruros* (**C**); the kidneys of a *Phylloscopus sibilatrix* (**B**); the intestine (**D**), gizzard (**E**), and heart (**F**) of a *Phoenicurus ochruros*; and the lungs of *Fringilla coelebs* (**G**–**I**). The developing megalomeronts (**A**,**E**,**F**) were surrounded by a capsular-like wall. The very young megalomeronts (**B**–**D**) were seen in cells with still the host cell nucleus present, which was not present in developing megalomeronts (**A**,**E**,**F**). Meronts in the lungs were seen either following the capillaries (**G**) or grouped tightly together (**H**,**I**) in a blood vessel of the lungs of *F. coelebs*. Cytomeres were readily visible in megalomeronts (**F**) as well as in developing and maturing meronts (**I**). The CISH signals were deep purple in the developing exo-erythrocytic stages (inserts and (**C**)). Long arrow: megalomeront; short arrow: capsular-like wall; arrowhead: host cell nucleus; opened arrowhead: meront. Scale bar: 25 µm (**A**–**D**,**F**–**I**); 100 µm (**E**).

3.2.2. Exo-Erythrocytic Stages Site of Development

Considering all the examined lineages of *H. majoris*, the kidneys and the gizzard were most commonly affected by megalomeronts, followed by the lungs and intestine, even though 10 birds had not their intestine and gizzard collected and investigated (Tables 1 and S1). By considering each lineage of the infection, the lungs, gizzard, and intestine were most often affected in hCCF5 infections, the gizzard in hCWT4 infections, the kidneys and lungs in hPARUS1 infections, and the heart and gizzard in hWW2 infections. In hPHSIB1 infections, only one individual was positive for megalomeronts, with the kidneys affected. In co-infections with several *H. majoris* lineages, the intestine and the brain were most commonly affected (Tables 1 and S1).

Very young megalomeronts were found in the brain (Figure 3C, co-infection of hWW2 and hPHSIB1), kidneys (Figure 3B, co-infection of hWW2 and hPHSIB1; Figures 2N and S3I,R,S,Z,AA, hPARUS1 infection), gizzard (Figures 2I and S2N, hCWT4), intestine (Figures S3D and S5H,I, co-infection hWW2 and hPHSIB1), pancreas (Figure 2D, hCCF5), and spleen (Figure S3T, hPARUS1 infection).

The number of *H. majoris* exo-erythrocytic stages present per organ varied per histological section analyzed. In most organs, megalomeronts were only seen sporadically, whereas, in the kidneys, pancreas, and gizzard, megalomeronts were found in greater numbers (up to 8 megalomeronts were seen in a single section) (Figures 2A,T, 3E, S2C and S3K,AD).

3.2.3. Seasonal Occurrence of Exo-Erythrocytic Stages of *H. majoris* hPARUS1

hPARUS1 lineage was sampled in spring, summer, and autumn and displayed exo-erythrocytic stages differently. Megalomeronts were seen in all seasons, however, with different frequencies (Table S2). In spring, the kidneys, lungs, liver, heart, intestine, and gizzard were found affected in 4 to 21 sections out of 13 to 31 sections for the 3 individuals investigated, while in summer megalomeronts were found in 1 to 5 sections out of 9 sections examined for one individual (Table S2). In autumn, mainly the kidneys were found to be affected (with the spleen found to be affected once in 1 bird only), with 21 sections positive for exo-erythrocytic stages out of 304 sections of kidneys investigated from 8 individuals (Table S2). Ten times more slides were thus investigated to find approximately the same number of positive sections for the kidneys between individuals collected in spring and autumn (Table S2). The other organs were not found affected by megalomeronts in autumn, even after investigating more than 100 sections, while in spring and summer, around 10 investigated sections reported a high number of exo-erythrocytic stages (Table S2).

In hPARUS1 single infections of *Haemoproteus*, very young megalomeronts were found in two individuals in spring and in four individuals in autumn. More of these very young megalomeronts were found in individuals in autumn (26 found in 21 positive sections out of 304 sections of the kidneys investigated) than in spring (3 found in 20 positive sections out of 31 sections of the kidneys investigated). In spring, more than 1 big megalomeront was found per section, adding up to more than 90 megalomeronts (without distinguishing if it is a different megalomeront from the previous section) in the kidneys in 20 positive sections out of 31, while in autumn, 11 big megalomeronts were found in the 304 investigated sections of kidneys.

3.2.4. Exo-Erythrocytic Stages in *H. majoris* Co-Infections with Several of Its Lineages

Five samples were found to be co-infected with several lineages of *H. majoris*, as determined by PCR-based testing and the obtained *cyt b* sequences (Tables 1 and S1). The megalomeront found in the brain of one individual co-infected with several *H. majoris* lineages (Figure 3A) could not be confirmed as *Haemoproteus* as the structure was absent in the CISH-tested section. Only eosinophilic staining and no basophilic staining was observed, which could indicate the occurrence of a degenerating megalomeront.

Very young megalomeronts were found in the brain, kidneys, and intestine of two individuals, each co-infected with hWW2 and hPHSIB1. The very young megalomeront found in the intestine was bigger (~96 μm in largest diameter) than the other very young

megalomeronts (<20 μm in largest diameter). However, the slightly enlarged host cell nucleus was still visible in the former (Figure 3D).

Some of the megalomeronts found in sample '20 Sp' co-infected with the lineages hPHSIB1 and hWW2 (Figure 3E) looked similar to those found in single infections with hCWT4 and hWW2 (Figure 2F,G,W,X).

3.3. Exo-Erythrocytic Stages of Other Haemoproteus species

Small meronts (up to 25 μm) were found in a *Fringilla coelebs* (sample '1 Sp') where microscopic examination identified the presence of *H. fringillae* in blood films. Positive CISH signals with the Haemo18*S* probe were observed for these meronts, confirming their *Haemoproteus* origin (Figure 3G insert). However, as no other sample investigated with single infection of *H. majoris* revealed small meronts, these stages likely belong to another *Haemoproteus* species, most likely *H. fringillae*, and not *H. majoris*.

Big meronts (up to 60 μm) were found in *F. coelebs* ('3 Sp') in a blood vessel of the lungs, and positive CISH signals were observed for the corresponding parasites seen in H&E-stained sections (Figure 3H,I). The meronts were tightly grouped in a blood vessel, forming a cluster well visible under the microscope. Individual meronts of this cluster were at different stages of development, with merozoites well visible in some meronts, which showed little CISH signal, whereas other developing meronts had deep purple CISH signals (Figure 3I insert). This indicates the asynchronous maturation of meronts and the asynchronous release of merozoites. Microscopic examination identified the presence of three other *Haemoproteus* species in the blood films of the *F. coelebs* individual '3 Sp' (*H. fringillae*, *H. magnus*, and *H.* sp.—Tables 1 and S1). It was not possible to determine the species identity of the *Haemoproteus* meronts found. However, as none of the *H. majoris* single-infected birds were found with such meronts, the latter are most likely not *H. majoris* but belong to one of the other *Haemoproteus* species present in the co-infection.

4. Discussion

The main result of this study is the report and comparative analysis of the exo-erythrocytic stages (megalomeronts) for five lineages (hCCF5, hCWT4, hPARUS1, hPHSIB1, and hWW2) of *H. majoris* in different avian host species. Megalomeronts developed in the examined bird individuals during single infections. This is the first comparative study that addresses the exo-erythrocytic development of different lineages of one *Haemoproteus* species in different avian hosts, providing opportunities to address possible patterns of development in avian haemoproteids. This study also identified the lineage hCWT4 as another lineage of *H. majoris*; this contributes to better understanding the intra-species genetic diversity of this widespread *Haemoproteus* species.

The lineages hPARUS1, hPHSIB1, hCCF5, and hWW2 were identified as *H. majoris* by Križanauskienė et al. [19], but the parasitemia intensity was too low in the sample infected with hCWT4 to describe and identify the species [19]. In our study, the microscopic examination of blood films of all birds revealed *H. majoris* infections, including all hCWT4 infections and the co-infections with several other *Haemoproteus* species.

Twelve birds were infected with *H. majoris* lineage hPARUS1, and megalomeronts were found to develop in the kidneys of eight, in the lungs of three, in the liver of two, and in the heart, spleen, pancreas, and gizzard of one bird (Tables 1 and S1). The megalomeronts were found in different bird species (*P. major*, *C. caeruleus*, and *P. montanus*) and their structure was similar to that of the megalomeronts previously reported in the kidneys, liver, lungs, and spleen of a single *P. major* [9]. This confirms the kidneys as the main site of development of *H. majoris* hPARUS1.

A previous report identified the kidneys and the intestine as sites of development of *H. majoris* lineage hPHSIB1 [12]. In our study, the lineage hPHSIB1 was found in three bird species, with megalomeronts observed only in the kidneys of one *P. sibilatrix* (Tables 1 and S1). It should be noted that the intestine and the gizzard were not collected for histological purposes for two individuals (*S. atricapilla* and *P. sibilatrix*), while the third individual (*Aegithalos caudatus*)

was found dead and no information on gametocytes was available. It is thus difficult to know if the intestine is one of the organs most commonly affected by hPHSIB1 infections or if this is only the case for the kidneys. No individuals were found infected with the lineage hPHYBOR04 of *H. majoris*, for which megalomeronts were described in the kidneys of a *Turdus pilaris* [9]. As was the case with lineage hPARUS1, only megalomeronts were found in all examined birds infected with the lineages hPHSIB1 (this study, [12]) and hPHYBOR04 [9].

H. majoris lineage hCCF5 was mostly found in *Fringilla coelebs*. Megalomeronts were found to develop mainly in the lungs, pancreas, and gizzard (Tables 1 and S1). Only one out of seven individuals showed megalomeronts in the kidneys. In this regard, hCCF5 seems different from the lineages hPARUS1, hPHSIB1, or hPHYBOR04, whose tissue stages mainly developed in the kidneys (this study, [9]). The morphology of the hCCF5 exo-erythrocytic stages was highly similar to the megalomeronts of the lineages hPARUS1, hPHYBOR04, and hPHSIB1 found in this and previous studies [9,12]. In all these megalomeronts, a capsular-like wall surrounded the parasite, in which interconnected cytomeres were seen to develop (Figure 2A–C).

H. majoris parasites of the lineages hCWT4 and hWW2 were mostly found affecting the gizzard (found in 5 individuals), even though the gizzard and intestine were not collected in 9 out of 15 birds. The kidneys were sampled and examined from all birds, but no megalomeronts were found during hCWT4 infections (eight individuals) and were seen only in one out of seven hWW2 infections. This differs compared with the previously reported sites of development of megalomeronts in hPARUS1, hPHSIB1, and hPHYBOR04 infections (this study, [9,12]). The morphology of some megalomeronts found in hCWT4 and hWW2 infections was slightly different from megalomeronts of other *H majoris* lineages. Mainly, the cytomeres were interconnected more tightly (similar to a spiderweb), while megalomeronts of hPARUS1, hPHSIB1, hCCF5, and hPHYBOR04 infections possessed interconnected cytomeres of a bigger size (compare Figure 2F,G,W,X with Figure 2A–C, Figures S2,S4 vs. S1,S3). This could reflect a lineage variation or a difference in the stage of development of the megalomeronts. The megalomeronts found in hCWT4 and hWW2 infections were more irregularly shaped compared with the typical roundish form of megalomeronts (compare Figure 2E–G,T–X with Figure 2A–C,J–M,O–R and Figures S2,S4 vs. S1,S3). This difference in shape could be due to the different locations in the organs. hCWT4 megalomeronts were found in the muscular layers of the gizzard, where they are exposed to varying pressures during peristaltic contractions of the organ. This could explain the irregular shapes of the megalomeronts observed. Megalomeronts in hCWT4 infections were also found to develop in the koilin cuticle of the gizzard, a thick protective layer on the inside of the gizzard that is continuously worn out and where no nutrients are delivered. This could be detrimental to the development of megalomeronts, as their growth might be altered. It is to be noted that none of the megalomeronts found in the koilin cuticle were mature (Figure 2E), and the CISH signals were only light purple, suggesting that the development of the parasite had stopped (low expression of RNA while the stage was not mature either).

Megalomeronts with fully developed merozoites showed barely any CISH signal (Figure S3P,AO), compared with the young and developing stages (Figures 2 and 3 vs. Figures S1–S5), a phenomenon already observed in other *Haemoproteus* parasites and suggesting low ribosomal content in mature stages [30]. The very young megalomeronts were found more easily due to their deep purple CISH signals, facilitating their search in the corresponding H&E-stained sections. Their small sizes and location in host cells with the host cell nuclei present but slightly enlarged were striking compared with previous reports of *Haemoproteus* parasites [7–13,15]. These very young megalomeronts were found in different host species infected with different *H. majoris* lineages (Figure 2D,I,N,S; Figures S3I,R–T,W–AA, S5H,I). In the infections with hPARUS1, very young megalomeronts were found in the kidneys of several individuals collected in different seasons. They were more often observed in autumn than in spring in positive sections, while developing and mature megalomeronts were more often found in spring.

Megalomeronts of *H. majoris* hPARUS1 were found in three bird species, two of which were found at different times of the year. *Parus major* were found infected with hPARUS1 twice in spring (one negative for megalomeronts in this study and one positive in [9]), once

in summer (positive in this study), and twice in autumn (positive in this study), while *Cyanistes caeruleus* was sampled twice in the spring (positive in this study) and five times in autumn (two positive and three negative in this study). The individuals sampled in spring contained the largest number of megalomeronts, with up to four organs affected, while fewer individuals were affected by megalomeronts in autumn, with only one to two organs affected (and at a lesser intensity). These seasonal differences in the maturation of the tissue stages might result from a different parasite-load strategy in the host. In Europe, spring is characterized by the active transmission of *Haemoproteus* parasites [1], and the increase in parasitemia in blood, which is readily visible in blood films. Mature megalomeronts are expected to be more numerous in spring, as merozoites are actively produced and released into circulation. In comparison, parasitemia usually markedly decreases in autumn and is barely present during winter. This seasonality in parasitemia is directly related to exo-erythrocytic development in haemoproteids and should result in less numerous megalomeronts in organs, lessening the burden on the host until the relapse of infection in the following spring [1]. It is still unknown under which stage (unicellular hypnozoite-like parasites or dormant megalomeronts at early development or advanced stage) *Haemoproteus* parasites are present in their host during winter, as gametocytes are rarely seen in blood films. A small number of young megalomeronts might be enough to initiate exo-erythrocytic development and relapse in spring, but their size and presence might also harm the host as inflammatory reactions are observed. Very young megalomeronts, due to their smaller size, might have a lesser influence on the host, but as their CISH signals were deep purple, active development and growth still occur. It is unknown whether the parasite can slow down its development and persist during winter.

Haemoproteus majoris is a generalist parasite [21] with six lineages differing by 1 to 6 bp (0.2–1.3 %) in the *cyt b* barcoding sequence. The lineage hCWT4 is closer to hWW2 and hPHYBOR04, with a 1 bp difference (0.2 %), whereas hPARUS1, hPHSIB1, and CCF5 have more differences from the other lineages (differences range from 2 to 6 bp). It seems probable that megalomeronts are the main and probably only tissue stage during *H. majoris* infections. The morphological similarities of megalomeronts and the sites of their development seem to reflect their genetic distances (hCWT4/hWW2 vs. hCCF5/hPARUS1).

This is the first study which analyzed more than two lineages belonging to one *Haemoproteus* species in regard to their exo-erythrocytic development. It is thus difficult to speculate if the slight differences in morphology and site of development between the *H. majoris* lineages are due to the generalist features of this parasite or are due to lineage-specific features, or both. It would be interesting to study additional multi-lineage species, such as *H. tartakovskyi*, *H. lanii*, and *H. balmorali*, to explore intraspecific variation of exo-erythrocytic development. Only one parasite closely related to *H. balmorali*, *H. attenuatus*, has been reported to develop meronts in the lungs of its host [14]. An investigation into the exo-erythrocytic stages of other closely related species, which are genetically closely related, would help to better understand if the genetic distances can predict patterns of the exo-erythrocytic development or if the development is only species-specific.

Differences in parasitemia intensity were small in most sampled birds, with parasitemia intensity mostly around 1% (Table 1). Among individuals infected with the same lineage and showing the same intensity of parasitemia, some contained megalomeronts but others did not (Table S1). Similar results were previously reported during *H. pastoris* infection in *Sturnus vulgaris* [13]. Mainly, no trend for megalomeront development was determined among the individuals with parasitemia ranging from 1 to 26%, with the exception of one individual with 10% parasitemia, for which no megalomeronts were found [13]. Parasitemia intensity was not associated either with the number of affected organs by megalomeronts. In *H. pastoris*, nine organs were reportedly affected by the parasites [13], while for all examined *H. majoris* lineages and hosts, megalomeronts were found in ten organs (this study, [9,12]).

Finally, two birds with *H. majoris* infections were found to be co-infected with other *Haemoproteus* spp., and other exo-erythrocytic stages were found. These exo-erythrocytic

stages were meronts of small sizes following the capillaries of the lungs (Figure 3G), and they were similar to meronts found in *H. dumbbellus* [30] of one individual or bigger and clustered together in a blood vessel of the lungs (Figure 3H,I) of the second individual. No meronts were previously reported in *H. majoris* infections, only megalomeronts (this study, [9,12]). Deep purple in situ CISH signals were observed in the meronts in the two individuals, confirming their *Haemoproteus* identity (Figure 3G–I inserts), and the microscopic examination of the blood smears identified co-infections in both cases with *H. fringillae*. It should be noted that the second individual was also infected with other *Haemoproteus* spp., aside from *H. majoris* and *H. fringillae*. It is most likely that these meronts are not of *H. majoris* but might very well be of *H. fringillae*. This observation shows the difficulties in the research on the exo-erythrocytic development of haemosporidians during co-infections, which predominate in wildlife and calls for the development of more specific in situ diagnostic probes (e.g., on a species or even lineage level), which would increase the resolution of exo-erythrocytic stages belonging to different parasite species.

5. Conclusions

This study extends knowledge about the lineage diversity of the widespread *H. majoris* haemosporidian parasite by adding the lineage hCWT4 to this morphospecies. Only megalomeronts were observed developing in all examined single *H. majoris* infections, strongly suggesting that meronts might be absent in this parasite. Megalomeronts were also found in mixed infections of several *H. majoris* lineages, while meronts were only found in cases of mixed infections of *H. majoris* and other *Haemoproteus* species. The following findings are worth more attention.

The organs were found to be differently affected by *H. majoris* megalomeronts, with hPARUS1 megalomeronts most commonly found in the kidneys; hCCF5 in the lungs, intestine, and kidneys; hCWT4 in the gizzard; and hWW2 in the heart and gizzard. Megalomeronts were also found in those organs for the other lineages (but at a lower intensity). The morphology of all reported *H. majoris* megalomeronts was mostly similar in all lineages, with some minor differences in hCWT4 and hWW2 infections. Very young megalomeronts were found for four of the *H. majoris* lineages. The parasitemia intensity could not be associated with the presence of megalomeronts in organs. However, megalomeronts were more often found in birds from spring than from autumn, with more developing and mature megalomeronts in spring and more very young stages in autumn. It would be interesting to investigate other generalist and specialist parasite species in different avian hosts across different seasons to better understand the pattern of their development and to make sure how the exo-erythrocytic development depends on the parasite lineage, the host species, and the season. It would also be interesting to see if very young megalomeronts can be found in the host during the winter period, possibly as dormant stages responsible for relapses in spring.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/pathogens12070898/s1. Figure S1: Megalomeronts of *Haemoproteus majoris* lineage hCCF5 in *Fringilla coelebs*; Figure S2: Megalomeronts of *Haemoproteus majoris* lineage hCWT4 in *Sylvia communis* and *Sylvia curruca*; Figure S3: Megalomeronts of *Haemoproteus majoris* lineage hPARUS1 in *Cyanistes caeruleus*, *Parus major*, and *Parus montanus*; Figure S4: Megalomeronts of *Haemoproteus majoris* lineage hWW2 in *Sylvia atricapilla*; Figure S5: Megalomeronts of *Haemoproteus majoris* found in co-infections with several of its lineages; Table S1: Data on all individuals with *Haemoproteus majoris* infections investigated for exo-erythrocytic stages; Table S2: Data on all individuals with *Haemoproteus majoris* hPARUS1 infections investigated for exo-erythrocytic stages numbers.

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