https://doi.org/10.15388/vu.thesis.710
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VILNIUS UNIVERSITY
NATURE RESEARCH CENTRE

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Bloodsucking dipterous insects (Culicidae, Ceratopogonidae): the biology and their role in transmission of blood parasites

DOCTORAL DISSERTATION

Natural Sciences,
Ecology and Environmental Sciences N 012

VILNIUS 2025

The dissertation was prepared between 2020 and 2024 at the Nature Research Centre.

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This doctoral dissertation will be defended in a public meeting of the Dissertation Defence Panel:
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The dissertation shall be defended at a public meeting of the Dissertation Defence Panel at 10:00 on 10th January 2025 in meeting room 101 of the Nature Research Centre.
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The text of this dissertation can be accessed at the libraries of the Nature Research Centre and Vilnius University, as well as on the website of Vilnius University:
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https://doi.org/10.15388/vu.thesis.710
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VILNIAUS UNIVERSITETAS
GAMTOS TYRIMŲ CENTRAS

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Kraujasiurbių dvisparnių vabzdžių (Culicidae, Ceratopogonidae) biologija ir jų vaidmuo kraujo parazitų pernešime

DAKTARO DISERTACIJA

Gamtos mokslai,
Ekologija ir aplinkotyra N 012

VILNIUS 2025

Disertacija rengta 2020–2024 metais Gamtos tyrimų centre.

Mokslinė vadovė – dr. Rasa Bernotienė (Gamtos tyrimų centras, gamtos mokslai, ekologija ir aplinkotyra, N 012).

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ABBREVIATIONS

BLAST – Basic Local Alignment Search Tool
BOLD – Barcode of Life Data System
bp – base pairs
CDC – Centers for Disease Control
COI – cytochrome c oxidase subunit 1
Cytb – mitochondrial cytochrome *b* gene
DNA – deoxyribonucleic acid
MalAvi – a database for avian haemosporidian parasites
MIR – minimal infection rate
NCBI – National Center for Biotechnology Information
PCR – polymerase chain reaction
RDA – Redundancy Analysis
rRNA – ribosomal ribonucleic acid
SE – standard error
SET – buffer, commonly used in molecular research (solution of Sodium Chloride, Ethylenediaminetetraacetic acid (EDTA) and Tris-HCl)
UV LED – ultra-violet light-emitting diode
VU – Vilnius University

TURINYS

INTRODUCTION

Blood-sucking insects play an extremely important role in ecosystems and human day-to-day life. They cause nuisance by biting and can transmit various pathogens, causing diseases, outbreaks, and killing hundreds of thousands of people every year. Diptera species are the most evolutionary advanced group of insects (Wiegmann et al., 2011). It is the second largest order of insects in terms of described species (Yeates and Wiegmann, 2005); it has the greatest diversity of blood-feeding insect species (Lehane, 2005). Diptera contains the following families of blood-sucking insects: Culicidae (mosquitoes), Ceratopogonidae (biting midges), Simuliidae (blackflies), Tabanidae (horseflies, deer flies), Hippoboscidae (louse flies), Psychodidae (sandflies), Muscidae (flies) (Lehane, 2005). Mosquitoes and biting midges are the most abundant insects amongst the bloodsucking Nematocera. The family Culicidae includes over 3.7 thousand species worldwide (Harbach, 2024), and a vast majority of mosquitoes feed on blood, but there are some mosquitoes (genus *Toxorhynchites* Theobald, 1901) that do not feed on blood and can survive on plant sugars (Collins and Blackwell, 2000). Four genera of Ceratopogonidae are known to feed on blood (*Forcipomyia* Meigen, 1818, *Leptoconops* Scuse, 1889, *Austroconops* Wirth and Lee 1958 and *Culicoides* Latreille, 1809). Genus *Culicoides* alone has roughly 1.4 thousand species worldwide (Borkent and Dominiak, 2020).

Natural vectors' investigation is important to understand better the transmission of parasite species in the wild, as well as for predicting possibilities of disease spread due to climate change and for the protection of endangered vertebrate species. Both, mosquitoes and biting midges can transmit parasitic protists from the orders Haemosporida and Trypanosomatida, which species are found worldwide and are often detected in birds in Lithuania. Haemosporida parasites can infect various animal groups, including amphibians, reptiles, birds, mammals (Valkiūnas, 2005). Avian haemosporidian parasites are widespread and can cause severe illness or even death in non-adapted bird species (Garnham, 1966; Ilgūnas et al., 2016). Studies of avian malaria serve as a model for better understanding of epidemiology of human malaria. In the past, studies of avian malaria helped to clarify the life cycle of haemosporidian parasites, develop *in vitro* cultivation techniques, advance chemotherapy, and contribute to knowledge on many other aspects of medical parasitology (Valkiūnas, 2005).

Research on avian blood parasites and their natural vectors as well as the transmission dynamics remain relatively scarce. So far, only in six European

biting midge species it was found that haemosporidian parasites were able to finnish their sporogonic development (Žiegytė et al., 2022), while nine mosquito species were proven experimentally to act as vectors for these infections (Ferreira et al., 2020; Santiago-Alarcon et al., 2012).

Trypanosomatids, particularly species of the genus *Trypanosoma* described by David Gruby in 1843, are notable parasites due to the severe diseases they cause in humans and domestic animals (Zídková et al., 2012). While extensively studied in these contexts, trypanosomes in wildlife, especially in birds, have received less attention despite evidence of some species being pathogenic. Avian trypanosomes are transmitted by insect vectors belonging to Hippoboscidae, Simuliidae, Culicidae, and Ceratopogonidae (Bernotienė et al., 2020; Molyneux, 1977; Votýpka and Svobodová, 2004). Data on mosquitoes and biting midges infected with *Trypanosoma* species is scarce, in various research 13 mosquito and seven biting midge species were found to be infected with *Trypanosoma* (Brotánková et al., 2022; Kazak et al., 2023; Miltgen and Landau, 1982; Molyneux, 1977; Votýpka et al., 2002; 2012).

Research of blood-sucking insects is crucial not only for disease prevention but also for understanding their biology, seasonality, ecological roles, and behavior, since those traits are related to their ability in pathogen transmission. These studies might as well help to identify potential bridge vectors – species that can transmit diseases across different host species, including humans, livestock, and wildlife (Truc et al., 2013). As the world becomes increasingly interconnected, the risks associated with these vectors grow, highlighting the importance of ongoing research. Additionally, understanding their ecology, life cycles, and feeding preferences is essential for developing effective control measures, especially in areas where these insects are endemic or where invasive species are becoming established. Another important aspect is climate change that can significantly influence the spread of invasive species and the parasites they transmit. As temperatures increase and weather patterns shift, some blood-sucking insects are expanding their ranges into new regions, increasing the risk of spreading diseases to previously unaffected areas.

While much is known about the medical and economic impacts of blood-sucking insects, knowledge gaps remain, particularly regarding their immature (larval) stages, certain aspects of their behavior, and their impact as vectors of pathogens in the wild. Research is also needed to explore the potential for these insects to spread emerging diseases or adapt to new environments, which could pose unforeseen risks to public health. While the

study of blood parasites is crucial, the role of blood-sucking insects as vectors cannot be overstated. These insects are important for public health, agriculture, and even global trade, making them a vital focus of entomological research. By deepening our understanding of these insects, we can better prepare for and mitigate the risks they pose in an increasingly globalized world.

OBJECTIVE AND MAIN TASKS OF THE STUDY

The objective was to evaluate the role of mosquitoes (Culicidae) and biting midges (Ceratopogonidae, *Culicoides*) in the transmission of avian blood parasites (Haemosporida (Apicomplexa) and Trypanosomatida (Euglenozoa)).

The following tasks were set to achieve the objective:

1. To reveal the mosquito larvae diversity, seasonality, and relationship between their abundance and certain physical and chemical parameters in various water bodies.
2. To determine mosquito species naturally infected with avian blood parasites (Haemosporida, Trypanosomatida) using integrative research approach.
3. To find out if hibernating *Culex pipiens* mosquitoes are infected with haemosporidian parasites and trypanosomes and compare these results with the data on mosquitoes collected during warm period of a year (twenty-four hours average temperature >10°C).
4. To determine biting midge species naturally infected with avian haemosporidian parasites (Haemosporida) using integrative research approach.

STATEMENTS TO BE DEFENDED

1. The presence and abundance of particular mosquito species depend on the temporality of water body, the bottom cover, the pH level, the NO_3^- concentration, and seasonality.
2. Mosquitoes of the genera *Coquillettidia, Culex,* and *Culiseta* have the the highest prevalence of haemosporidian parasites. Species of genus *Ochlerotatus* has the lowest infection prevalence of haemosporidian parasites.
3. *Culex pipiens* is a likely natural vector of *Plasmodium matutinum* lineage pLINN1, as this parasite completes sporogonic development and its sporozoites are present in salivary glands of this wild-caught mosquito.
4. Hibernating *Culex pipiens* mosquitoes are found to be not infected with haemosporidian and *Trypanosoma* parasites, only with monoxenous trypanosomatids. Active *C. pipiens* mosquitoes are infected with avian (*Trypanosoma culicavium*), mammalian (*T. trinaperronei, T. theileri*) trypanosomes, and avian haemosporidian parasites.
5. *Culicoides pictipennis, C. segnis,* and *C. kibunensis* biting midges have the highest prevalences of haemosporidian parasites.
6. *Culicoides segnis* is a likely competent vector for *Haemoproteus fringillae* hCCF3, *H. majoris* hPHSIB1, *H. asymmetricus* hTUPHI01, and *H. minutus* hTURDUS2. *Culicoides kibunensis* is a likely competent vector for *Haemoproteus belopolskyi* hHIICT1, *H. homominutus* hCUKI1, *H. parabelopolskyi* hSYAT01 and hSYAT02, and *Haemoproteus* sp. hSYAT13. *Culicoides reconditus* is a likely natural vector of *Haemoproteus magnus* hROFI1. *Culicoides pictipennis* is a likely competent vector of *Haemoproteus parabelopolskyi* hSYAT01, *Haemoproteus* sp. hSYAT13, *H. homogeneae* hSYAT16, *H. asymmetricus* hTUPHI01, and *H. minutus* hTURDUS2, while *Culicoides festivipennis* is a likely competent vector of *Haemoproteus belopolskyi* hHIICT1.

NOVELTY OF THE STUDY

1. *Culex pipiens* mosquitoes were proven to be likely natural vectors of *Plasmodium matutinum* lineage pLINN1 as sporozoites of *P. matutinum* pLINN1 were microscopically detected in mosquito salivary glands for the first time.
2. *Plasmodium ashfordii* (pGRW02) was molecularly detected in bloodsucking insect (*Ochlerotatus sticticus*) for the first time. Previously, it was reported only in birds. This also shows that this mosquito naturally feed on birds' blood.
3. *Trypanosoma trinaperronei* was found in *Culex pipiens* mosquitoes for the first time.
4. For the first time, sporozoites of five genetic lineages of haemosporidian parasites were found in *C. kibunensis*, five lineages in *C. pictipennis*, four lineages in *C. segnis*, and one in *C. festivipennis* biting midges.
5. For the first time, sporozoites of genetic lineages hCUKI1, hCULPIC02, hROFI1, hSYAT01, hSYAT13, hSYAT16 of haemosporidian parasites were found in salivary glands of biting midges showing vectorial capacity of these insects.
6. Sporozoites of *H. magnus* (hROFI1) were found in *C. reconditus* salivary glands for the first time.
7. Hibernating *Culex pipiens* were not infected with haemosporidian parasites and thus can be recommended for experimental research of avian malaria using the natural population of wild-caught (not reared in laboratory) insects.

1. LITERATURE OVERVIEW

1.1. Mosquitoes (Culicidae)

Mosquitoes belonging to the family Culicidae (order Diptera; suborder Nematocera) are well-known for their ability to feed on blood, causing a nuisance for people and other animals. Their notoriety can also be attributed to their plasticity and adaptability to various environmental conditions, enabling their worldwide distribution (Foster and Walker, 2019).

Ever since Linnaeus described the first mosquito species in the 10th edition of Systema Naturae (1758), the list consisted of merely six *Culex* Linnaeus, 1758 species. This list included now well-known and studied species such as *Culex pipiens* Linnaeus, 1758 (common name: Northern house mosquito), *Culex bifurcatus* (now agreed to be a heterotypic synonym for *C. pipiens* (Harbach, 1985)), *Culex pulicaris* (now known as a species belonging to the Ceratopogonidae family – *Culicoides pulicaris* (Linnaeus, 1758), *Culex reptans* and *Culex equinus* (both now recognized as members of the Simuliidae family: *Simulium reptans* (Linnaeus, 1758) and *Simulium equinus* (Linnaeus, 1758), and *Culex stercoreus* (now considered nomen dubium). Currently, there are 3726 known mosquito species worldwide, grouped into two subfamilies (Anophelinae and Culicinae) and 113 genera (Harbach, 2024). Thirty-seven mosquito species have been identified in Lithuania (Bernotienė and Lučiūnaitė, 2011; Pakalniškis et al., 2006), belonging to six genera: *Aedes* Meigen, 1818, *Anopheles* Meigen, 1818, *Coquillettidia* Dyar, 1905, *Culex*, *Culiseta* Felt, 1904, and *Ochlerotatus* Lynch-Arribalzaga, 1891.

Some mosquito species are grouped into species complexes. At certain life cycle stages (usually adult), some sibling species can be hard to distinguish but can differ in larval stage, behavioral patterns, and ecology. *Anopheles claviger* (Meigen, 1804) was shown by Coluzzi with colleagues in 1960–1965 to be part of a complex with the same name (Anopheles claviger complex) (Becker et al., 2003). Among the species found in Lithuania, only *A. claviger* has been identified so far (Pakalniškis et al., 2006). The Anopheles maculipennis complex was distinguished in the 20s and 30s of 20th century by Falleroni, Martini, Van Thiel, Rivista, and Missiroli, and recognized in 1940 by Bates. Species of this complex morphologically look very similar, but crossbreeding experiments, cytotaxonomy, and enzyme electrophoresis conducted by Stegnii and Kabanova in 1976, Bullini and Coluzzi in 1978, and Suzzoni-Blatger and colleagues in 1990 showed that these species are reproductively isolated (Becker et al., 2003). Anopheles maculipennis

complex species can be identified by the structure of their eggs (White, 1978), although some differences were found between species in adult and larval stages. However, intraspecific variation exists, making it necessary to investigate a series of specimens as some features can be lost (scales can fall from mosquitoes' body during flight or because of the friction). Species found in Lithuania belonging to this complex are *Anopheles atroparvus* Van Thiel, 1927, *Anopheles maculipennis* Meigen, 1818, and *Anopheles messeae* Falleroni, 1926 (Pakalniškis et al., 2006).

Two species found in Lithuania, *Aedes cinereus* Meigen, 1818 and *Aedes geminus* Peus, 1970, are not grouped into a complex, although they are very similar and can be identified by hypopygial characters of males. Bernotienė and Lučiūnaitė (2011) determined that both of these sibling species can be found in Lithuania.

Among *Ochlerotatus* species, there are multiple species groups with distinctly similar characteristics. The following groups of *Ochlerotatus* species are based on publications written by Gutsevich and colleagues in 1974, Martini in 1931, Mohrig in 1969, and Natvig in 1948, (Becker et al., 2003):

- The annulipes group: *Ochlerotatus annulipes* (Meigen, 1830), *Ochlerotatus behningi* (Martini, 1926), *Ochlerotatus cantans* (Meigen, 1818), *Ochlerotatus cyprius* (Ludlow, 1919), *Ochlerotatus euedes* (Howard, Dyar, and Knab, 1912), *Ochlerotatus excrucians* (Walker, 1856), *Ochlerotatus flavescens* (Müller, 1764), *Ochlerotatus riparius* (Dyar and Knab, 1907).
- The caspius group: *Ochlerotatus caspius* (Pallas, 1771), *Ochlerotatus dorsalis* (Meigen, 1830).
- The communis group: *Ochlerotatus cataphylla* (Dyar, 1916), *Ochlerotatus communis* (De Geer, 1776), *Ochlerotatus nigrinus* (Eckstein, 1918), *Ochlerotatus leucomelas* (Meigen, 1804).
- The intrudens group: *Ochlerotatus diantaeus* (Howard, Dyar, and Knab, 1912), *Ochlerotatus intrudens* (Dyar, 1919), *Ochlerotatus pullatus* (Coquillett, 1904).
- The punctor group: In Lithuania, only one species from this group is found so far – *Ochlerotatus punctor* (Kirby, 1837).

The genus *Culex* includes the *Culex pipiens* species complex, which was suggested to be called the Pipiens assemblage by Harbach in 2012. This assemblage includes *C. pipiens pipiens*, its biotype *Culex pipiens molestus* Forskal, 1775 (previously described as a separate species), *Culex pipiens quinquefasciatus* Say, 1823, *Culex pipiens pallens* Coquillett, 1898, *Culex*

restuans Theobald, 1901, and *C. p. pipiens* sibling species *Culex torrentium* Martini, 1925.

The genus *Culiseta* was previously known as *Theobaldia* Neveu-Lemaire, 1902. The name had to be changed due to nomenclature rules, as there was already a mollusk genus by the same name since 1885 (Becker et al., 2003).

Coquillettidia is a genus with one species that can be found in Lithuania – *Coquillettidia richiardii* (Ficalbi, 1889) (Pakalniškis et al., 2006). At first it was named *Culex richiardii* later *Coquillettidia* was placed as a subgenus of *Mansonia* Blanchard, 1902 by Stone in 1963 (Nugroho et al., 2020), but during the same year Ronderos and Bachman suggested to separate *Mansonia* and *Coquillettidia* genera. In the Catalogue of Mosquito in the World these two genera are treated as separate ones (Knight and Stone, 1977).

1.1.1. Culicidae species morphology

Adult mosquitoes have one very easily distinguishable feature among all Nematocera members: a long and scaled proboscis that is longer than the thorax (Figure 1.1.1.1.). The proboscis extends forward along with the maxillary palps (Rueda, 2008). When describing females, the length of the maxillary palps is significant, especially identifying *Anopheles*. The color and distribution of scales on the proboscis and palps can aid in species identification. In males, the palps are typically as long as or longer than the proboscis in most species (Becker et al., 2003). Mosquito males have plumose antennae, while females have pilose antennae (Figure 1.1.1.2.). Mosquitoes' antennae begin with a basal segment (scape), while the second, enlarged segment (pedicel) covers it (Wood et al., 1979). The pedicel has a well-developed mechanoreceptor and sound receptor (Johnston's organ). The remaining 13 flagellomeres form a flagellum. Most of the mosquito's head is occupied by compound eyes (Marshal, 1938; Snodgrass, 1959).

The thorax of mosquito comprises of three segments: the prothorax, mesothorax, and metathorax like other insects. Another organ within the mosquito thorax is a pair of three-lobed salivary glands (Snodgrass, 1959). Like all Diptera insects, mosquitoes have only forewings, which are used for flying, and thus the mesothorax is the most developed segment. Mosquitoes have narrow, well-developed wings with a specific pattern of veins, which are covered along the veins and margins with scales (Ross and Horsfall, 1965). The color, shape, and distribution of these scales can assist in mosquito species

identification. The hind wings are modified into halters, which act as gyroscopic organs. The dorsal side of the mosquito's thorax is shielded by the scutum. The scutum is usually covered with scales that can vary in color, shape, and pattern. This feature can be important in species identification but may not always be helpful since scales often fall off while mosquitoes are in

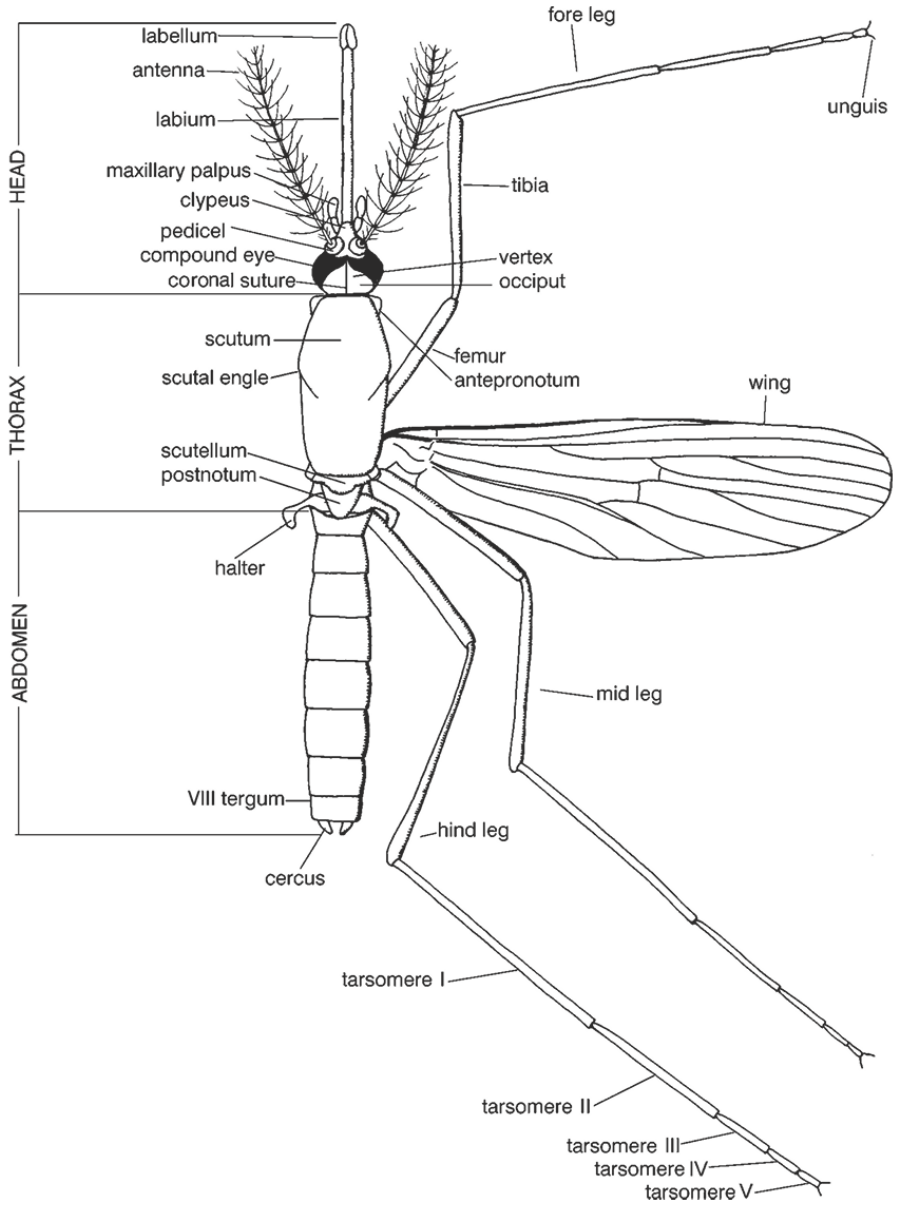

Figure 1.1.1.1. General culicine mosquito female's body plan (Marshall, 1938).

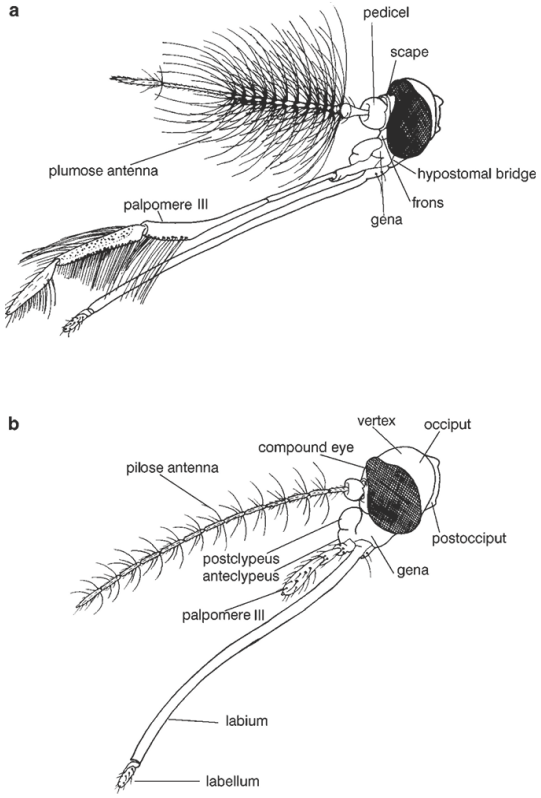

Figure 1.1.1.2. Heads of culicine mosquitoes: a) male‘s; b) female‘s (Wood et al., 1979).

flight. Mosquitoes have two pairs of spiracles on their thorax (mesothoracic and metathoracic) (Figure 1.1.1.3.) and the areas surrounding the mesothoracic spiracle are crucial for genus identification (Becker et al., 2003). Lateral thoracic segments can have setae, scales, and various combinations of these features, which can aid in species identification. Mosquito legs are covered with scales and may have rings or stripes on various segments (Foster and Walker, 2019).

The abdomen of mosquitoes consists of eleven segments. The last abdominal segments are reduced or modified to facilitate mating, egg-laying, and defecation. Pregenital segments (in males II–VIII and in females II–VII) are well developed. Segments I–VII on the lateral sides have pairs of spiracles. The dorsal side of the pregenital segments is covered by tergites, and the coloration and distribution of scales can help in species identification. For identifying male mosquitoes, a major feature is the form of various genital structures. In cases where female mosquitoes cannot be fully identified and molecular identification is not possible, identifying males can be crucial (Becker et al., 2003; Foster and Walker, 2019).

Mosquito larvae develop in water and have a completely sclerotized head capsule, a thorax composed of three fused segments, and an abdomen with ten segments. The combination of labral brushes, the expanded thorax, and the siphon helps to distinguish mosquito larvae from those of other Diptera (Foster and Walker, 2019). The head structure of Culicinae and Anophelinae mosquito larvae is similar, but their head shapes differ in both subfamilies

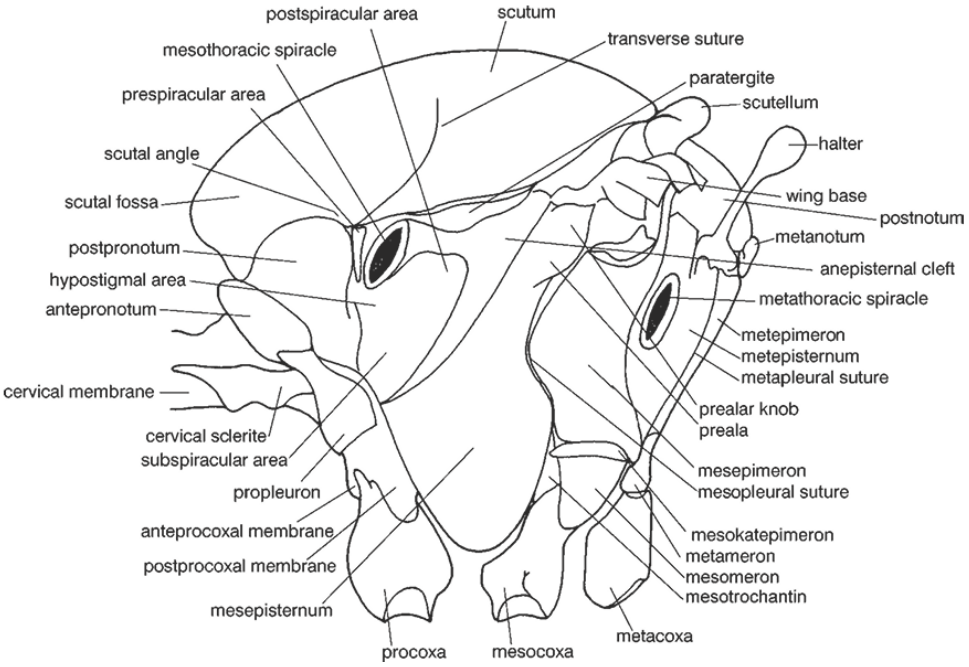

Figure 1.1.1.3. A side view of mosquitoes‘ thorax (Becker et al., 2003).

(Gunay et al., 2018). Another important feature for identifying mosquito larvae is the antennae (sensory appendages), which can be straight or slightly curved, vary in texture, and may have setae. Mosquito larvae also have a pair of compound eyes and stemmata (simple larval eyes) positioned just behind the compound eyes (Snodgrass, 1959).

The thorax of mosquito larvae is broader than both the head and abdomen. It is completely or mainly membranous and has up to 15 pairs of setae (numbered from 0-P to 14-P). Of these, only the setae in positions 1-P to 3-P are typically used in the identification process (Becker et al., 2003). The abdomen of mosquito larvae is comprised of ten segments. The first seven segments are very similar to each other and the VIII–X segments are different. The I–VII segments have setae which specific number and arrangement are used in identification. For instance, in *Anopheles* species, certain setae are palmate, while in other genera like *Culex*, the number of branches on certain setae (e.g., setae 1 on segments III–V) is a diagnostic feature (Rueda, 2008). Same segments can have sclerotized tergal plates (*Anopheles* larvae, segments I–VII feature sclerotized tergal plates, which are absent in most *Culex* species except for a few). The VIII segment has a lot of scales in a structure called the comb. Form of those scales, shape of their arrangement, and number can be one of identification features. This segment also has spiracles for respiration

that runs through a siphon. Characteristics like the siphonal index (ratio of siphon length to its basal width) (Gunay et al., 2018), the number and arrangement of siphonal setae (1-S), and the structure of pecten teeth (spiny projections) are crucial for species differentiation (Barr, 1958) (Figure 1.1.1.4.). The IX segment is reduced and can be visible in some species as a small ring at the base of X segment. The X segment is smaller and bears a saddle-shaped plate, which varies in shape among species. The ventral brush or fin made up of setae 4-X is used for locomotion and is another key identification feature, as is the length and structure of the anal papillae involved in osmoregulation (Becker et al., 2003).

Mosquito pupae, often called "tumblers," have a characteristic comma shape. They have a cephalothorax with the abdomen curled underneath. A pair of air trumpets on the dorsal mesothorax allows the pupa to breathe at the water's surface. Inside the cephalothorax, the developing adult appendages (head and thorax) are visible. The eighth abdominal segment has two broad paddles used for movement, allowing the pupa to propel itself by flexing its abdomen (Foster and Walker, 2019). Their abdominal structures such as setae and paddles can be used in identification, but it is rarely done (Becker et al., 2003).

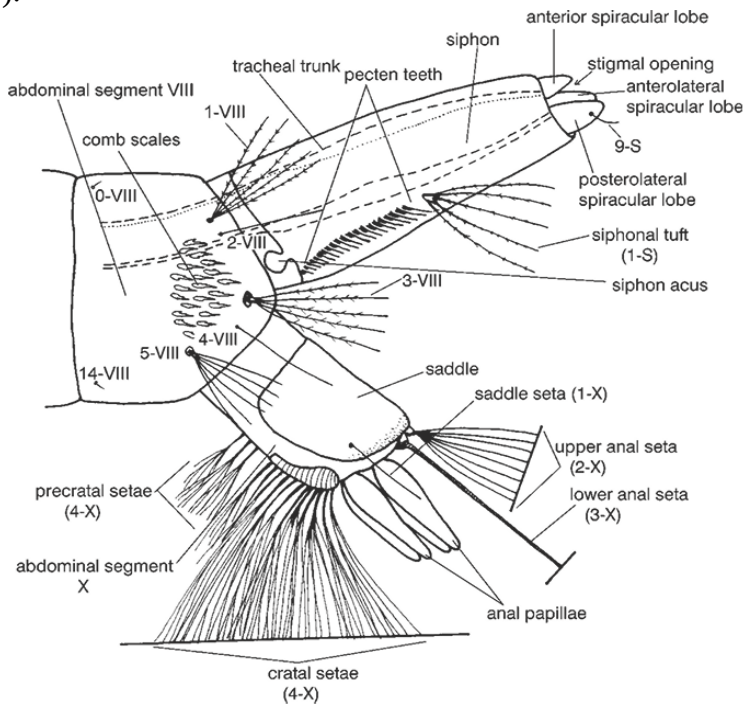

Figure 1.1.1.4. Culicine mosquito VIII-X segments of abdomen (Becker et al., 2003).

1.1.2. Brief description of mosquitoes' biology

Mosquitoes undergo complete metamorphosis, which includes egg, larval, pupal, and adult stages (Rueda, 2008). Water is crucial for the development of Culicidae mosquitoes, but they can develop in various aquatic environments (Wegner, 2009a). They can thrive in permanent or temporary water bodies; some species prefer clean water habitats, while others can develop in salty or polluted waters (Becker et al., 2003). Ecologically mosquitoes can be divided into floodwater, snow-melt, and tree-hole species, and some mosquitoes can develop in man-made water containers such as rainwater drums, old tires, and various pots (Foster and Walker, 2019; Rueda, 2008).

Depending on the species and temperature, mosquito females can lay 50–200 eggs two to four days after a blood meal (Becker et al., 2003). The genus *Anopheles* lay single eggs on the water surface, while other genera lay eggs in batches. For example, *Culex* mosquitoes lay several hundred eggs in rafts that float on the water surface, and *Coquillettidia* lay their eggs in radial batches attached to underwater plants (Foster and Walker, 2019; Lehane, 2005).

The embryos of mosquitoes that lay eggs on the water surface do not enter diapause and start their development right away, and these mosquitoes usually overwinter in the adult stage (Barr, 1958). Such eggs are not resistant to desiccation, so present water and not-too-cold temperatures are essential for their successful development (Becker et al., 2003). Mosquitoes that overwinter in the egg stage lay their eggs in moist areas or on damp substrates, and these eggs are well-adapted to desiccation and can remain viable for months or, in some cases, years until they receive a stimulus for hatching (stagnant water, decline in dissolved oxygen levels) (Barr, 1958). This remarkable adaptation ensures that they choose areas that will eventually be flooded and have minimal mosquito predators (Quiroz-Martinez and Rodriguez-Castro, 2007).

Depending on how many gonotrophic cycles a mosquito female has, they can be categorized as univoltine (producing only one generation per year, e.g., *Ochlerotatus excrucians*, *O. flavescens*, *O. pullatus*, and some other species that produce only one generation per year in their northern-most habitats (Becker et al., 2003)), bivoltine (producing two generations per year, e.g., *Ochlerotatus berlandi* (Séguy, 1921), *Ochlerotatus mercurator* (Dyar, 1920) – these species were not found in Lithuania (Pakalniškis et al., 2006)), and multivoltine (producing more than two generations per year, e.g., *Aedes*

cinereus, *Aedes geminus*, and many species in their southern habitats (Becker et al., 2003).

Mosquito larvae are active, representing the stage where they feed and grow between molts (mosquito larvae have four instars). Since Anophelinae mosquitoes do not have a siphon, they keep their body horizontal to the water surface when breathing. In contrast, Culicinae mosquitoes hang head down from the water surface, breathing through the siphon, with an exception: *Coquillettidia* mosquitoes, which have a modified siphon that can pierce submerged parts of aquatic plants and breathe oxygen from plant aerenchyma (Wilkerson et al., 2021).

The length of mosquito larval development is temperature-dependent, and the optimum temperature varies among different species. For example, *Culex pipiens* larvae develop in 7–8 days at a water temperature of 30°C, but it takes 48–58 days at 10°C (Becker et al., 2003). Mosquito larvae can develop in a wide range of temperatures. The optimum temperature depends on the species, but overall, the speed of development is temperature dependent. The process of larval development will be longer at lower temperatures and shorter at higher temperatures (Wilkerson et al., 2021).

The mosquito larvae diet consists of various microorganisms, algae, invertebrates, protozoans, and detritus. Some mosquito larvae are filter or suspension feeders, browsers, or predators (though predatory larvae are not found in Europe) (Pucat, 1965). When disturbed, mosquito larvae can dive for a short period (Wilkerson et al., 2021).

After larval development, the pupal stage follows, which is also aquatic and mobile, but at this stage, the pupae do not feed. Metamorphosis takes place during the pupal stage when some of the larval organs are histolyzed, and the adult mosquito body starts to form (Foster and Walker, 2019). When resting, pupae float at the water surface with respiratory trumpets protruding for respiration. However, when disturbed, pupae can dive quickly with the help of abdomen movements and paddles (Foster and Walker, 2019). Since *Coquillettidia* mosquitoes breathe oxygen from plant aerenchyma, their pupae develop underwater. When development is complete, the pupae must float to the water surface thus the tips of their pupal trumpets must break off so the pupa can be released from the plant to the water surface (Snodgrass, 1959).

The pupal stage can last from two days in warm water, and for all mosquito species, it takes longer in lower water temperatures. When the time comes for the adult to emerge, the pupa's abdomen straightens, and the ingestion of air causes a split in the cephalothorax, allowing the adult to rise up. At this stage, mosquitoes are extremely susceptible to disturbances,

especially strong winds that can cause them to fall into the water. Right after emergence, mosquitoes can fly short distances, but longer flights are possible after sclerotization (Foster and Walker, 2019). Typically, adult male mosquitoes emerge roughly two days earlier than females due to shorter larval development, a longer period of reaching sexual maturity (the male's hypopygium must rotate 180° before they are ready to mate) than females (Becker et al., 2003).

Emerging adults have some nutrients (lipids, glycogen) carried over from the larval stage, which act as an energy source for the first few days. Later on, both males and females feed on sugar from plants (nectar, honeydew) (Takken and Verhulst, 2013). Normally, the energy obtained from the first sugar meal is used for sexual maturation, mating, dispersal, and, for females, finding an appropriate blood meal (Foster and Walker, 2019). Typically, females mate only once (in a span of 24–48 h), while males can mate multiple times. After mating, female mosquitoes start their search for a blood meal (Takken and Verhulst, 2013). Feeding on and digesting vertebrate blood (with a few exceptions in some species or populations where the female can lay eggs without a blood meal) initiates egg development if the meal is large enough and the female is gonoactive (ovarian follicles reach the resting stage) (Becker et al., 2003; Foster and Walker, 2019).

Because of the caused nuisance, we are highly aware that many mosquitoes can feed on humans, however, many mosquito species can feed on wild mammals, birds, amphibians, and reptiles (Becker et al., 2003). Mosquitoes tend to favor specific host species, but some species can be less selective when there are no favorable food sources available (Boundenga et al., 2016, 2017; Takken and Verhulst, 2013). Blood-feeding starts with appetitive searching, meaning that hunger drives the mosquito to fly without orientation, which might bring them into contact with a potential host (Lehane, 2005). The majority of mosquito species seek hosts during dusk and dawn. During this time-of-day temperature is dropping and relative humidity is increasing (high temperatures and direct sunlight cause mosquitoes to lose moisture). Such mosquitoes are more active during the nights with moonlight (Bidlingmayer, 1964). Mosquito species that fly extensively can show two ways of non-oriented flight – passive, meaning that they drift with the wind and other way – active dispersal (Becker et al., 2003). After locating a potential host, mosquitoes switch their behavior to activation and orientation, beginning an oriented search based on host stimuli. The final stage, attraction, brings mosquitoes close to the host, allowing them to evaluate the suitability of the potential host for feeding (Lehane, 2005; Sutcliffe, 1987). Various

authors managed to measure great distances that *Aedes vexans* (Meigen, 1830) mosquitoes can fly from their emergence habitats – 22 km (Clarke, 1943) and even up to 48 km (Becker et al., 2003; Gjullin et al., 1950). Although not all mosquito species show such impressive results in distances that they can fly. Snow-melt species have a tendency to keep those distances short and to seek blood-meals and suitable places for egg laying closer to their emergence place. *Ochlerotatus communis* were caught flying up to 1.6 km from their breeding habitats and *A. cinereus* flying less than 800 m away from their breeding sites (Nielsen, 1957).

Visual cues can be important for mosquitoes in locating a potential host, but olfaction is one of the most important sensory components of blood-seeking insects (Becker et al., 2003; Foster and Walker, 2019). Carbon dioxide is one of the main compounds that mosquitoes use to locate their blood meal, especially in the activation and orientation stage. Carbon dioxide receptors are located in mosquitoes' palps and can sense tiny changes (+0.01%) in CO_2 concentration, helping them locate the source. Other olfactory stimuli include lactic acid, fatty acids, ammonia, phenolic components of urine, acetone, butanone, indole, and 6-methyl-5-hepten-2-one (Lehane, 2005). Sensilla coeloconica located on mosquitoes' antennae helps to sense other important stimuli such as heat (Davis and Sokolove, 1975).

During cold or dry seasons mosquitoes can enter hibernation and wait for a more suitable time. In the temperate climate zone mosquitoes' hibernation period normally is winter. *Aedes* and *Ochlerotatus* mosquitoes in temperate climate diapause in the egg stage (larvae do not hatch until suitable conditions appear). Some species (*Coquillettidia richiardii*, *Anopheles claviger*, *Anopheles plumbeus*, and sometimes *O. rusticus* and *Culiseta morsitans* (Theobald, 1901) can hatch in the autumn and overwinter in second-third larval stage) can hibernate in larval stage (if it occurs, their metabolism is reduced and this way larval development is delayed until favorable conditions occur) (Becker et al., 2003). *Culex, Culiseta*, and *Anopheles* mosquitoes in temperate climate zone are known to overwinter as adults in various shelters that do not have frost. Such hibernating mosquito females usually do not feed on blood during winter and rather use the accumulated fat body as an energy source. Some studies about *Culex pipiens* noted about their inability to use blood as a source for lipid reserves for hibernating (Mitchell and Briegel, 1989), other studies determined that 40% of hibernating female *C. pipiens* did not undergo ovarian maturation after they were subjected to warmer temperature period and took full blood meals which gave them an equal

chance of surviving (Eldridge and Bailey, 1979). There is evidence of blood-feeding in diapausing *C. pipiens* females in the wild (Bailey et al., 1982).

1.1.3. Culicidae role in ecosystems and animal health

The first and most noticeable effect of mosquitoes is the discomfort and nuisance caused by their bites, which can impact real estate values, the tourism industry, outdoor activities, and even result in losses of domestic animals (Schmidt and Roberts', 2009; Steelman, 1976). Although with over 3700 mosquito species, only a fraction bother or bite people. Mosquitoes play several roles in ecosystems: larvae while feeding filtrates water, they serve as food for aquatic organisms, adults provide nourishment for terrestrial animals (birds, bats, amphibians, and lizards), mosquitoes act as pollinators (Bhattacharya et al., 2016; Fang, 2010). Mosquitoes themselves can be used for vector control – predatory larvae of genus *Toxorhynchites* Theobald, 1901 can feed on other mosquito larvae, which can be used in water bodies, where chemical control is not possible (Collins and Blackwell, 2000). Mosquitoes can possibly influence the vertebrate regulation proceses, because of their ability to transmit pathogens and influence the natural selection process (Bhattacharya et al., 2016).

The role of mosquitoes as both ecological contributors and major public health threats has provoked extensive research into their biology and control (Becker et al., 2003; Floore, 2006; Lehane, 2005). As early as 1877, Patrick Manson demonstrated that filarial worms are transmitted by mosquitoes (Chernin, 1983). Two decades later, in 1897, Ronald Ross discovered that culicine mosquitoes are involved in the transmission of avian malaria. In 1898, Giovanni Battista Grassi and his colleagues showed that human malaria is transmitted by anopheline mosquitoes (Cox, 2010). In 1901, Walter Reed and James Carroll proved that yellow fever is transmitted by mosquitoes. Mosquitoes were the first arthropods to be identified as intermediate hosts for vertebrate parasites. Mosquitoes transmit a huge variety of viruses belonging to the families Togaviridae, Flaviviridae, Bunyaviridae. Amongst best known vertebrate viruses transmitted by mosquitoes are Eastern, Western and Venezuelan equine encephalomyelitis, Chicungunya, O'nyong nyong, Ross River, Yellow fever, Dengue, Japanese encephalitis, St. Louis encephalitis, Muray Valley encephalitis, West Nile, Zika (Foster and Walker, 2019). Besides malaria causing parasites, filarial worms and various arboviruses, mosquitoes can transmit trypanosomes (Schoener et al., 2018).

Furthermore, the nuisance caused by mosquitoes during blood-feeding and the damage they inflict on livestock and wildlife (McCook, 1889) raise the question, "Can the Mosquito Be Exterminated?" At that time McCook noted "…The question is easier asked than accurately answered…". However, nowadays the importance of mosquitoes in ecosystems has led researchers to carefully consider the potential consequences of their eradication. While eliminating disease-carrying species could save countless lives and boost economic development, particularly in regions like sub-Saharan Africa, scientists are also aware that other organisms could fill the ecological niches left behind, potentially creating new challenges (Bhattacharya et al., 2016).

The ongoing battle against mosquitoes highlights the complexity of balancing public health goals with ecological conservation. Although the eradication of certain mosquito species might lead to short-term relief from human suffering, the long-term impacts on ecosystems remain uncertain. As researchers continue to explore the roles of mosquitoes, they must weigh the benefits of eradication against the potential ecological problems left behind (Fang, 2010).

1.2. Biting midges (genus *Culicoides*)

The family Ceratopogonidae comprises of more than 6200 species, grouped into approximately 123 genera. Among these, the genus *Culicoides* is the most significant in terms of species that can transmit various pathogens while feeding on the blood of vertebrates, including humans (Mullen and Murphree, 2019). The genus *Culicoides* includes 1347 extant and 52 fossil species (Borkent and Dominiak, 2020), with 29 species currently recorded in Lithuania (Bernotienė et al., 2023; Pakalniškis et al., 2006). Like mosquitoes, biting midges are distributed worldwide, except in Antarctica and New Zealand (Mellor et al., 2000).

As mentioned above, the genus *Culicoides* for the first time was put on scientific-taxonomic map "disguised" as *Culex* mosquitoes by Linnaeus (1758). Such confusion might have happened because of their feeding habits, although their mouth parts, overall bodily features, and body size differs heavily from mosquitoes'. Genus *Culicoides* was described in 1809 by Latreille.

Genus *Culicoides* is divided to 33 subgenera (Borkent and Dominiak, 2020), while in Lithuania there are seven of them (Bernotienė et al., 2023; Pakalniškis et al., 2006):

- *Avaritia* Fox, 1955: *Culicoides chiopterus* (Meigen, 1830); *Culicoides obsoletus* (Meigen, 1818); *Culicoides scoticus* Downes and Kettle, 1952;
- *Beltranmyia* Vargas 1953: *Culicoides circumscriptus* Kieffer, 1918; *Culicoides salinarius* Kieffer, 1914; *Culicoides sphagnumensis* Williams, 1955;
- *Culicoides*: *Culicoides deltus* Edwards, 1939; *Culicoides fagineus* Edwards, 1939; *Culicoides grisescens* Edwards, 1939; *Culicoides impunctatus* Goetghebuer, 1920; *Culicoides newsteadi* Austen, 1921; *C. pulicaris* (mentioned earlier that this species was first identified by Linnaeus (1758) as mosquito *Culex pulicaris*); *Culicoides punctatus* (Meigen, 1804);
- *Monoculicoides* Khalaf, 1954: *Culicoides stigma* (Meigen, 1818); *Culicoides nubeculosus* (Meigen, 1830); *Culicoides riethi* Kieffer, 1914;
- *Oecacta* Poey, 1853: *Culicoides albicans* Winnertz, 1852; *Culicoides festivipennis* Kieffer, 1914; *Culicoides heliophilus* Edwards, 1921; *Culicoides kibunensis* Tokunaga, 1937; *Culicoides pictipennis* (Staeger, 1839); *Culicoides simulator* Edwards, 1939; *Culicoides vexans* (Staeger, 1839);
- *Silvaticulicoides* Glukhova, 1972: *Culicoides achrayi* Kettle and Lawson, 1955; *Culicoides fascipennis* (Staeger, 1839); *Culicoides pallidicornis* Kieffer, 1919; *Culicoides subfascipennis* Kieffer, 1919;
- *Wirthomyia* Vargas, 1973: *Culicoides reconditus* Campbell and Pelhan-Clinton, 1960; *Culicoides segnis* Campbell and Pelhan-Clinton, 1960.

Similarly, as with Culicidae mosquitoes some species of *Culicoides* biting midges are grouped into species complexes. These species can be difficult to identify morphologically in adult female stage. More well-known species complexes are Culicoides obsoletus complex (species *C. obsoletus* and *C. scoticus* – these species females can only be distinguished molecularly (Pages and Sarto I Monteys, 2005)), Culicoides pulicaris complex (species *C. pulicaris, C. punctatus, and C. impunctatus* (Groschupp et al., 2023)), and Culicoides chiopterus complex (with only one species in Lithuania known so far – *Culicoides chiopterus*) (Meiswinkel et al., 2004).

1.2.1. *Culicoides* species morphology

Adult *Culicoides* midges are small, usually measuring 1–2.5 mm in body length. Female *Culicoides* feed on vertebrate blood and therefore have short well-developed mouthparts for piercing and blood feeding. The female's mouthparts are surrounded by a proboscis, formed by the extension of the labium-epipharynx, bladelike mandibles (with a row of teeth for lacerating the skin), maxillae, and a hypopharynx with a median groove where saliva passes during feeding (Mullen and Murphree, 2019) (Figure 1.2.1.1.). Males have reduced mouthparts and do not feed on blood (Szadziewski et al., 1997). A key taxonomic feature is the pair of maxillary palps, which is a five-segmented structure where the third segment is enlarged and contains a sensory pit. The shape of the third segment and the sensory pit (or in some species multiple pits) are useful for species identification. The antennae of males and females differ, male antennae are plumose (feather-like), while female antennae are

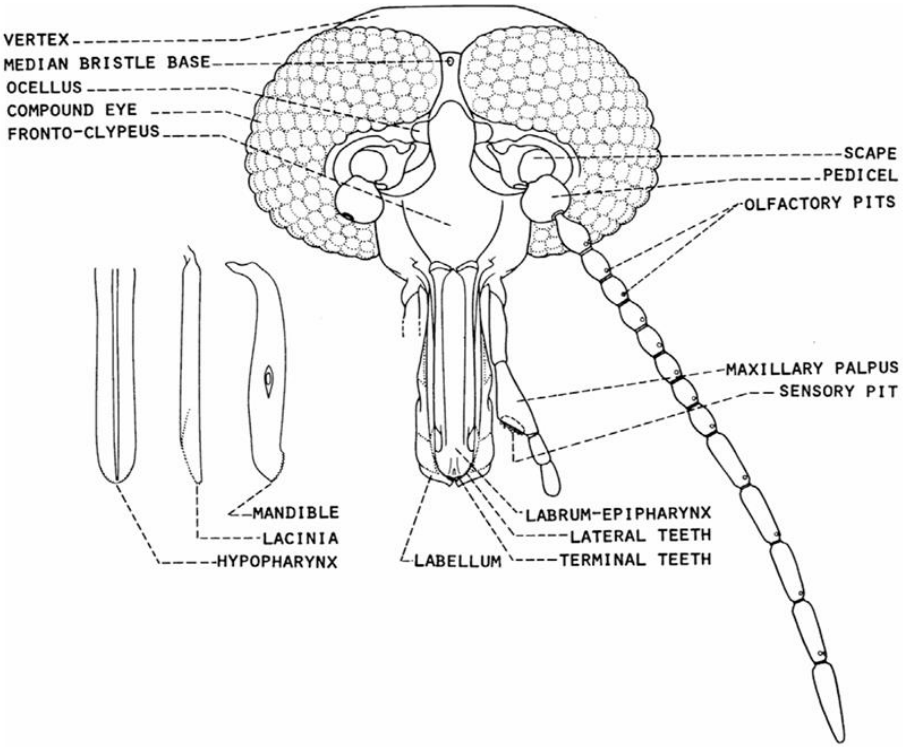

Figure 1.2.1.1. Morphology of female *Culicoides* biting midge head and it‘s mouthparts and antennae (Blanton and Wirth, 1979).

pilose. Both antennae are 15-segmented, consisting of a basal scape, an enlarged pedicel containing a Johnston's organ, and 13 flagellomeres. The segments have varying numbers of small sensory pits (sensilla coeloconica), which are important taxonomic characters (Mullen and Murphree, 2019; Szadziewski et al., 1997).

The wings of *Culicoides* have distinctive patterns and are of great taxonomic importance therefore wings help in species identification (Figure 1.2.1.2.). Wings have specific shape and specific venation. While distinguishing *Culicoides* biting midges from other ceratopogonids, the morphology of two similar-sized radial cells in most species can help. In a lot of cases there are patterns of white and dark spots that can be used for species identification. When resting, midges lay their wings flat against the abdomen. The number of spermatheca is also an important feature while identifying *Culicoides* species. It varies between one to three (Boorman, 1993).

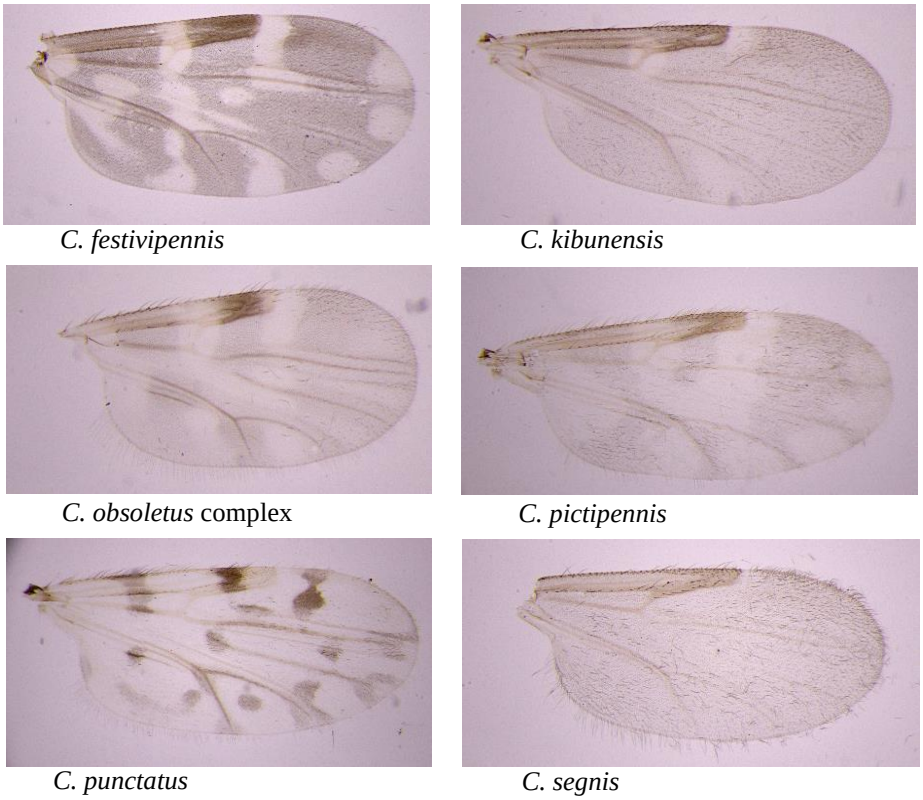

Figure 1.2.1.2. Examples of *Culicoides* wing patterns. Specimens were collected in Lithuania. (Original, photo K. Valavičiūtė-Pocienė).

Unlike in the case of mosquitoes, it is possible to identify parous *Culicoides* females without dissection. A burgundy-red pigment which forms in the wall of abdomen during the development of the ovarian follicles can be observed (Dyce, 1969) (Figure 1.2.1.3.). This feature can be used for identification and sorting females that have already passed a gonotrophic cycle and had at least one bloodmeal, this way increasing the probability of detecting blood parasites in collected females if investigating only parous females.

Figure 1.2.1.3. *Culicoides pictipennis* female specimens: a – engorged; b – parous; c – nuliparous. White arrow indicates burgundy pigment while black arrow shows blood meal in the abdomen. (Original, photo M. Kazak).

Culicoides larvae are typically long and slender with a white or translucent body, measuring 2 to 5 mm in length at the fourth instar. They have a yellow-to-brown head capsule, behind which is the thorax with subcutaneous pigmentation forming a pattern (Mullen and Murphree, 2019). Although, *Culicoides impunctatus* has a distinctly darker muddy brown head (Kettle and Lawson, 1952). The thoracic and abdominal segments are similarly sized (Szadziewski et al., 1997). *Culicoides* larvae lack distinctive setae, except for four pairs at the caudal end. Additionally, they possess a pair of anal papillae used for osmoregulation, which are usually retracted into the rectum. Larvae generally rely on cutaneous respiration. Depending on the species, their mouthparts are adapted for scraping, tearing, or seizing (Mullen and Murphree, 2019). Their mandibles are not opposable; they move vertically or rotate during feeding. Despite the medical and economic importance of *Culicoides*, effective morphological species identification systems for their larvae are still lacking (Yanase et al., 2013). Biting midge larvae species can be identified only at their fourth instar. The fourth (final) stage larvae can be recognized by hollowed out meso- and metathoracic lateral bodies. Fourth

instar larvae can also be measured by the length of their head – at this stage it is longer than 250 μm. Just before the pupation fourth instar larvae starts exhibiting pupal features like respiratory horns and the caudal spines. At this time larvae become so distorted that any efforts of identification can get very difficult (Kettle and Lawson, 1952).

Culicoides pupae are typically brownish and feature a pair of relatively short prothoracic respiratory horns at the anterior end, with numerous tiny spiracular openings at the tips (Szadziewski et al., 1997). These tubes repel water, allowing aquatic forms to remain at the water surface and access air during metamorphosis. An air pocket beneath the developing wings provides additional buoyancy (Mullen and Murphree, 2019).

1.2.2. Brief description of *Culicoides* biology

Culicoides biting midges can inhabit a variety of environments, including marshes, swamps, coastal areas of various water bodies (lakes, ponds, rivers), bogs, peatlands, tree holes, and other moist or semi-aquatic areas or moist substrates (like animal dung, rotting fungi, wood), as moisture is essential for their development (Szadziewski et al., 1997). This huge variety of habitats lets them live in all climate zones and continents, with Antarctica and New Zealand being exceptions (Mellor, et al., 2000). Rainfall is closely related to larval density (Kettle, 1956). Normally in temperate climates *Culicoides* biting midges are active in summertime, but in the tropics, they are present all year round (Lehane, 2005). Their life cycle consists of four stages: egg, larva, pupa, and adult (Mullen and Murphree, 2019; Szadziewski et al., 1997) (Figure 1.2.2.1.). *Culicoides* full developmental cycle at an optimal temperature of 20–25°C lasts around one month (Gutsevich, 1973).

Adult *Culicoides* females typically require a blood meal to develop their eggs, although some are autogenous. If they have sufficient nutrients from the larval stage, they may produce eggs in their first gonotrophic cycle without a blood meal (Boorman and Goddard, 1970). *Culicoides* eggs are small and elongated, measuring 250–500 μm in length, and are covered with minute projections. Initially white, the eggs turn brown over time (Szadziewski et al., 1997). Egg development depends on temperature and moisture, typically taking 7–10 days to hatch, though it can be as short as 2–3 days under optimal conditions (*Culicoides nubeculosus*) in room temperature hatched after 48–65 hours (Lawson, 1951). Females lay eggs in batches on moist substrates; depending on the species and the size of the blood meal, a batch

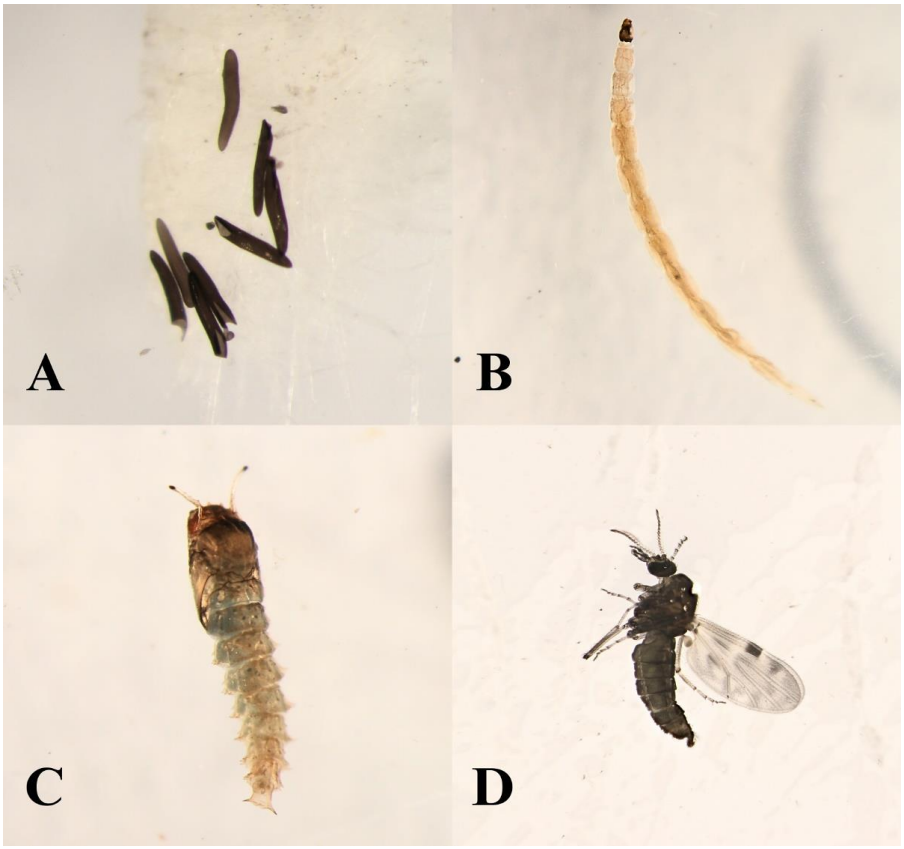

Figure 1.2.2.1. *Culicoides* biting midge developmental stages: A – eggs; B – larvae; C – pupae; D – adult female. (Original, photo A,B,C – K. Valavičiūtė-Pocienė; D – M. Kazak).

can consist of 30 to over 450 eggs. Depending on species, biting midges can lay their eggs singly, in loose groups, string, or in masses coated with gelatinous substance (Szadziewski et al., 1997)

Culicoides larvae develop from two weeks to a year, depending on the season, species, and latitude. In experimental research of *C. nubeculosus* by Glukhova in 1989 it was determined that in 28–30°C larval development took 22–32 days (I instar – 4–5 days, II – 3–4, III – 7–11, and VI – 8–11 days). Larvae prefer organically rich substrates, and prolonged drought with high temperatures can be unfavorable, potentially killing the larvae. Biting midge larvae can feed on microorganisms in water, they can even be cannibalistic and eat other biting midge larvae. They can consume algae, decaying organic matter, protozoans, bacteria (Boorman, 1974; Szadziewski et al., 1997). Many species spend around 7–8 months as larvae. *Culicoides* larvae that live in

water are good swimmers and are recognized by their snake-like, side-to-side movements (Mullen and Murphree, 2019).

Larvae pupate near the surface of the substrate, allowing their pupal prothoracic horns to reach and penetrate the water film. Species that inhabit water-filled tree holes have pupae that float at the water surface but adhere loosely to the sides of the tree cavity. Most species overwinter as III, VI instar larvae and pupate in spring or early summer (Szadziewski et al., 1997). Like mosquitoes, male biting midges emerge before females to prepare for mating. Unlike mosquitoes, however, their genital structures rotate temporarily 180° rather than permanently (Mullen and Murphree, 2019). During mating, males gather in swarms near water or in open areas close to suitable breeding sites, usually not far away from their own emergence sites since they can be quite specific (Mellor et al., 2000). Females enter these swarms, where males identify them by wing-beating frequencies and copulation occurs if the female is receptive. If females do not require a blood meal, they lay eggs within a week of emergence. Development of each batch of eggs typically requires a blood meal. Overall, all life stages of biting midges are sensitive to the changes in their environment (Werner et al., 2020). Anautogenous females can lay eggs after a blood meal. Following a blood meal, biting midge females seek shelter in vegetation to rest for a few days until the eggs develop (Szadziewski et al., 1997).

Both males and females feed on plant nectar to get energy for the flight. Only females feed on vertebrate blood, with their food sources varying by species (Szadziewski et al., 1997). Some species prefer to bite birds, while others target different groups of mammals, reptiles, or amphibians. Some biting midges are host-specific, while others are generalists, feeding on various animal groups based on availability (Kazak et al., 2024). Female mouthparts are adapted to lacerate the skin and capillaries with serrated mandibles, causing blood to pool at the site of the lesion. The midge then draws the blood into the foregut and midgut using a pharyngeal pump (Mullen and Murphree, 2019). Feeding occurs mostly in the evening and the beginning of the night (Lehane, 2005). Biting midge adults remain close to suitable habitats for egg-laying, avoiding strong winds and benefiting from high humidity, which can prolong their lifespan and allow more females to successfully mature their eggs (Szadziewski et al., 1997).

1.2.3. *Culicoides* role in ecosystems and animal health

Biting midges have various roles in nature. They contribute to ecosystem health through nutrient cycling (while larvae are feeding on organic matter, algae, fungi, etc.), supporting food webs (all biting midge life stages can become food for insectivorous animals), and playing occasional roles in pollination (while feeding on plants' sugars) (Boorman, 1993; Mullen and Murphree, 2019).

Biting midges cause nuisance to humans, livestock, and wild animals, they can cause pain and allergic reactions (Blackwell and King, 1997). Because of their small size they can fly around and into ears, nose, and eyes (Durden and Mullen, 2019). Since biting midges are so small, they can be overlooked (no wonder they received their common name "no-see-ums") and because of that mosquitoes are often blamed for *Culicoides* bites (Mullen and Murphree, 2019).

Big population densities, widespread distribution, and feeding behavior of biting midges make them suitable vectors for various pathogens (Meiswinkel et al., 2004; Mellor et al., 2000). They can transmit arboviruses (bluetongue, epizootic hemorrhagic, African horse sickness) (McDermott and Lysyk, 2020), various protozoan parasites like hemosporidians (genera *Haemoproteus* Kruse, 1890, *Leucocytozoon* Berestneff, 1904) (Valkiūnas, 2005), trypanosomatids (Svobodová et al., 2017), and nematodes (Žiegytė et al., 2021).

Globalization, active traveling helps biting midges (sometimes even infected ones) to be introduced to new territories. This could become very harmful if pathogens harbored by competent biting midges are transmitted to naïve local fauna (Tatem et al., 2012). Even though biting midges can spread dangerous pathogens, research on their ecology, larval and pupal morphology is still lacking. Mainly this is due to difficulties in rearing them in laboratory conditions (Yanase et al., 2013).

1.3. Protozoan parasites transmitted by mosquitoes and biting midges

1.3.1. Haemosporida parasites

Haemosporidian parasites (phylum Apicomplexa) infecting birds are transmitted by blood-sucking insects and are divided into four genera: *Plasmodium* Marchiafava and Celli, 1885, *Haemoproteus, Fallisia* Lainson,

Landau, and Shaw, 1974, and *Leucocytozoon*. Parasites of species belonging to these genera have similar life cycles (some differences occur), and they use dipteran insects where sexual process and sporogony occur. Species of genus *Plasmodium* are transmitted by mosquitoes, and species of genus *Haemoproteus* are transmitted by *Culicoides* biting midges and louse flies (Hippoboscidae) (Valkiūnas, 2005).

Before molecular methods were developed, experimental studies were key in proving vectorial capacity of mosquito species (Table 1.3.1.1.). The beginning of the 21st century is notable for the development and extensive application of molecular techniques that have helped to alleviate further haemosporidian parasite research by adding supplementary sensitive diagnostic tools (Bensch et al., 2000; Martinsen et al., 2008; Perkins et al., 2008; Perkins and Schall, 2002; Ricklefs and Fallon, 2002; Santiago-Alarcon et al., 2010). The MalAvi database (Bensch et al., 2009; accessed June 19, 2024) was created in order to compile molecular data, and it lists 282 entries on *Plasmodium* parasites (115 genetic lineages) in Culicidae mosquitoes.

Table 1.3.1.1. Experimental studies which prove mosquito vectorial capacity for some Plasmodium species. The table is based on the information provided by Santiago-Alarcon et al., 2012; Ferreira et al., 2020.

Mosquito species	***Plasmodium* species**	**References**
Culex pipiens	*Plasmodium relictum* (Grassi and Feletti, 1891)	Huff, 1927; Hunninen, 1951, 1953; Kazlauskienė et al., 2013; Neumann, 1908; Ruge, 1901; Sergent and Sergent, 1907; Tate and Vincent, 1934; Valkiūnas et al., 2015; Žiegytė et al., 2014
	P. cathemerium	Carlson et al., 2016
	Plasmodium subpraecox Grassi and Feletti, 1892	Raffaele, 1932
	Plasmodium giovannolai Corradetti, Verolini, and Neri, 1963	Corradetti et al., 1963a,b
	Plasmodium durae Herman, 1941	Valkiūnas, 2005

Mosquito species	***Plasmodium* species**	**References**
Culex pipiens	*Plasmodium garnhami* Guindy, Hoogstraal, and Mohammed, 1965	Garnham, 1966
	Plasmodium vaughani Novy and MacNeal, 1904	Corradetti and Scanga, 1972
	Plasmodium rouxi Sergent, Sergent, and Catanei, 1928	Huff, 1932; Manwell, 1947
	Plasmodium kempi Christensen, Barnes. and Rowley, 1983	Christensen et al., 1983
	Plasmodium elongatum Hartman, 1927	Huff, 1927; Micks, 1949; Raffaele, 1934
	Plasmodium juxtanucleare Versiani and Gomes, 1941	Akiba, 1959
Culex quinquefasciatus	*P. relictum*	Valkiūnas et al., 2015
	P. cathemerium	Carlson et al., 2018
Culex stigmatosoma Dyar, 1907	*P. cathemerium*	Carlson et al., 2016; 2018
Culex tarsalis Coquillett, 1896	*P. cathemerium*	Carlson et al., 2018
Culex territans Walker, 1856	*P. rouxi*	Huff, 1932
	P. elongatum	Huff, 1927
Ochlerotatus communis	*P. relictum*	Koch, 1899
Ochlerotatus geniculatus (Olivier, 1791)	*Plasmodium gallinaceum* Brumpt, 1935	Roubaud et al., 1939

Mosquito species	*Plasmodium* species	References
Culiseta annulata (Schrank, 1776)	*Plasmodium circumflexum* Kikuth, 1931	Corradetti, 1964; Reichenow, 1932
Culiseta morsitans	*Plasmodium polare* Manwell, 1934	Meyer and Bennett, 1976
	P. circumflexum	Meyer et al., 1974; Meyer and Bennett, 1976
	P. vaughani	Williams and Bennett, 1978

The majority of mosquito species that are vectors of avian malaria belong to the genera *Culex*, *Aedes*, and *Culiseta*. Around 60 mosquito species were proven experimentally to be able to act as vectors for avian malaria parasites (LaPointe et al., 2012). *Plasmodium* species are known to infect humans and non-human primates (Cox, 2010), rodents (Vaughan and Kappe, 2012), reptiles (Perkins and Austin, 2009), and birds (Valkiūnas, 2005).

In vertebrate hosts, exoerythrocytic merogony (primary and secondary), erythrocytic merogony, and gametocyte formation occur. If a mosquito feeds on an infected bird, mature gametocytes round up and escape from erythrocytes within minutes. Subsequently, macrogametes and microgametes fuse to form a zygote, which develops into an ookinete (Valkiūnas, 2005). Ookinetes in the mosquito's midgut move to midgut epithelial layer, reach the basal lamina, and transform into oocysts, which are round and covered by capsule-like walls of host origin. Mature oocysts of *P. relictum* are often about 40 μm but can grow larger (Kazlauskienė et al., 2013). This stage is readily visible on the midgut surface under a microscope. Sporogony occurs in oocysts, producing hundreds of sporozoites. The duration of sporogony depends on temperature, parasite species, genetic lineage, and other factors. In *Culex pipiens*, the sporogony of *P. relictum* at 24°C is completed seven days after ingesting infected blood. After mature oocysts rupture, the sporozoites enter the mosquito's hemocoel and migrate to the salivary glands (Valkiūnas, 2005) (Figure 1.3.1.1.). If the vector species is suitable for development of a parasite and abiotic conditions are favorable, sporozoites reach the salivary glands, and the mosquito can infect another vertebrate during feeding (Valkiūnas et al., 2015). This underscores the importance of studying the feeding habits, reproductive characteristics, and the impact of parasitic organisms on mosquitoes' behavior.

In 1957, Fallis and Wood demonstrated that biting midges could transmit *Haemoproteus nettionis*, and in 1960, Akiba showed that

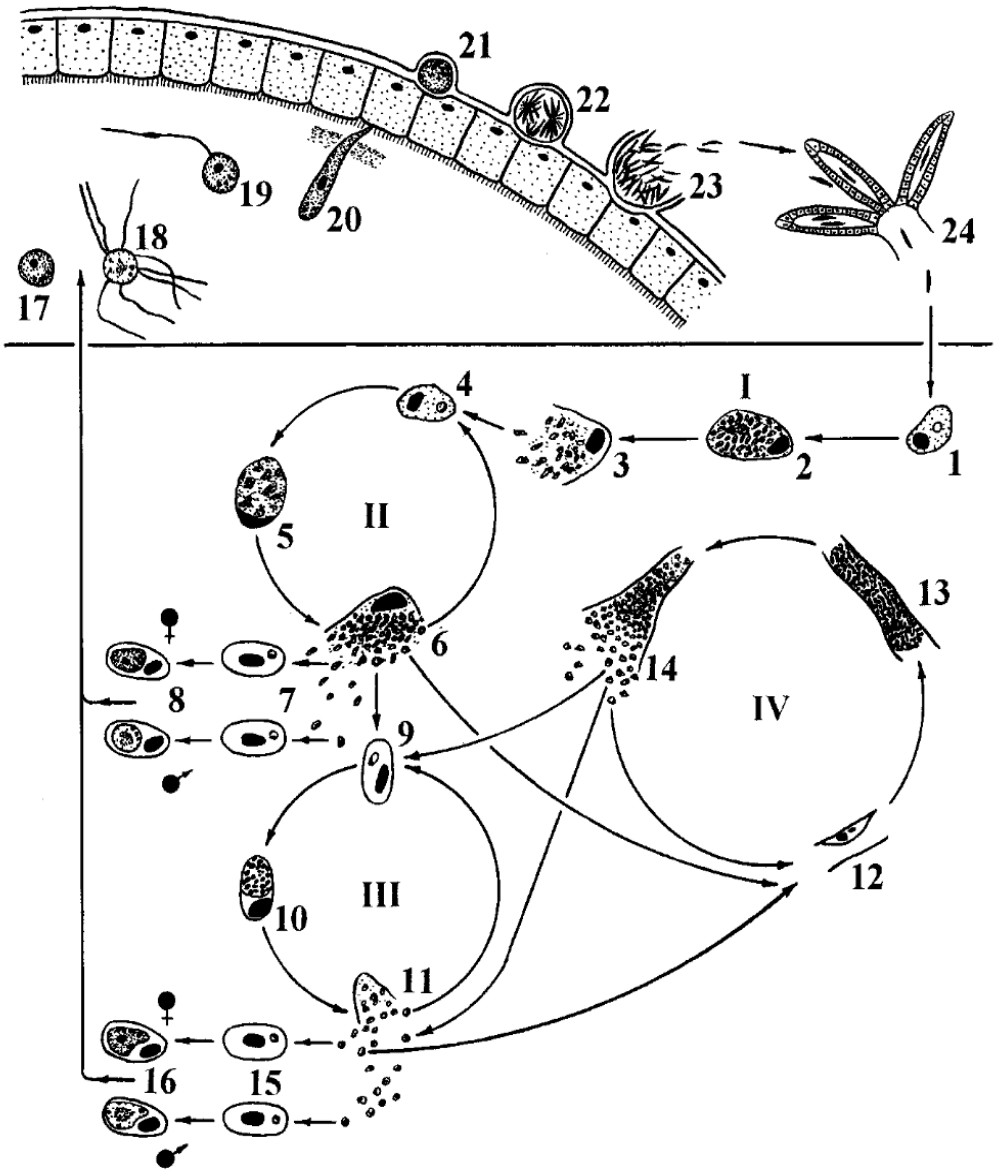

Figure 1.3.1.1. Diagram of life cycle of bird malaria parasites. Lower part, in bird: I, II – primary exoerythrocytic merogony; III – erythrocytic merogony; IV – secondary exoerythrocytic merogony; 1 – sporozoite in reticuloendothelial cell; 2, 3 – cryptozoites; 4 – merozoite in macrophage; 5, 6 – metacryptozoites; 7 – merozoites in erythrocytes; 8 – gametocytes; 9 – merozoite in erythrocyte; 10, 11 – erythrocytic meronts; 12 – merozoite in endothelial cell of capillaries; 13, 14 – phanerozoites; 15 – merozoites in erythrocytes; 16 – gametocytes. Upper part, in insect vector: 17 – macrogamete; 18 – exflagellation of microgametes; 19 – fertilization of macrogamete; 20 – ookinete penetrating the peritrophic membrane; 21 – young oocyst; 22, 23 – sporogony; 24 – sporozoites in the salivary glands of vector (Valkiūnas, 2005).

biting midges are vectors of *Leucocytozoon caulleryi* (Santiago-Alarcon et al., 2012). These were the first examples of *Culicoides* biting midges being identified as vectors for haemosporidian parasites. Further, there were studies proving experimentally which biting midge species can transmit which *Haemoproteus* parasites (Table 1.3.1.2.).

Table 1.3.1.2. Experimental studies which prove biting midge vectorial capacity for *Haemoproteus* species. The table is based on the information given in Bukauskaitė et al., 2015, 2019; Chagas et al., 2018; Santiago-Alarcon et al., 2012; Žiegytė et al., 2017.

Biting midge species	***Haemoproteus* species**	**References**
C. sphagnumensis	*Haemoproteus mansoni* Castellani and Chalmers, 1910	Fallis and Bennett, 1960
	Haemoproteus velans Coatney and Roudabush, 1937	Khan and Fallis, 1971
	Haemoproteus fringillae Labbé, 1894	Valkiūnas, 1997; 2005
	Haemoproteus danilewskii Kruse, 1890	Bennett and Fallis, 1960; Fallis and Bennett, 1961
C. impunctatus	*H. fringillae*	Valkiūnas, 1997; 2005; Valkiūnas and Iezhova, 2004a
	Haemoproteus parabelopolskyi Valkiūnas et al., 2007	Valkiūnas and Iezhova, 2004b
	Haemoproteus lanii Mello, 1936	Valkiūnas and Iezhova, 2004a
	Haemoproteus balmorali Peirce, 1984	Valkiūnas et al., 2002; Žiegytė et al., 2017
	H. dolniki	Valkiūnas et al., 2002
	Haemoproteus tartakovskyi Valkiūnas, 1986	Valkiūnas et al., 2002

Biting midge species	*Haemoproteus* species	References
C. impunctatus	*Haemoproteus noctuae* Celli and Sanfelice, 1891	Bukauskaitė et al., 2015
	H. majoris	Žiegytė et al., 2017
	Haemoproteus motocillae Bennett and Perice, 1990	Žiegytė et al., 2017
	H. pallidus	Žiegytė et al., 2017
C. nubeculosus	*Haemoproteus handai* Maqsood, 1943	Miltgen et al., 1981
	H. noctuae	Bukauskaitė et al., 2015
	Haemoproteus syrnii (Mayer, 1910)	Bukauskaitė et al., 2015
	H. minutus	Bukauskaitė et al., 2019
	H. motacillae	Bukauskaitė et al., 2019
	Haemoproteus attenuates Valkiūnas, 1989	Bukauskaitė et al., 2019
	Haemoproteus homopalloris Chagas et al. 2018	Chagas et al., 2018

Typically, *Haemoproteus* are found in birds but can also be present in reptiles (Garnham, 1966). Initially, these parasites were often considered as being benign in vertebrates, which resulted in limited attention to their pahogenicity. However, recent data indicates that these parasites can cause serious diseases due to damage of various internal organs, and even lead to mortality in infected individuals (Valkiūnas and Iezhova, 2022).

Currently, there are 177 *Haemoproteus* species that can be identified morphologically (Valkiūnas and Iezhova, 2022). According to the MalAvi database (accessed June 19, 2024), there are 2024 genetic lineages of *Haemoproteus*. Research on haemosporidian parasites genetic diversity in vertebrate hosts is more extensive, with 6393 records of different genetic lineages from avian hosts in the MalAvi database. In contrast, there are only 101 records of *Haemoproteus* detected in biting midges. Most of these recent records come from studies where parasites were detected molecularly, without microscopy reports of sporozoites in the salivary glands of investigated insects. To date, sporogonic development in biting midge salivary glands have

been identified for only six *Haemoproteus* species (seven genetic lineages) (Bernotienė et al., 2019; Žiegytė et al., 2021, 2022, 2023).

1.3.2. Trypanosomatida parasites

Trypanosomatids (phylum Euglenozoa) are protozoan flagellates that parasitize a wide range of organisms, from humans to plants (Schmidt and Roberts, 2009). They belong to the class Kinetoplastea, which is characterized by the presence of a kinetoplast, a specialized structure containing a large amount of DNA within a single mitochondrion. Approximately 400 species of trypanosomatids have been described (Maslov et al., 2013). Trypanosomes are said to be one of the most ancestral groups of protists (Lukeš et al., 2014).

Trypanosomatids exhibit two types of life cycles (Maslov et al., 2013; Simpson et al., 2006): dixenous life cycle (the parasite alternates between a vertebrate host and the digestive tract of a blood-sucking invertebrate, e.g. *Trypanosoma, Leishmania* Ron.Ross, 1903, and *Phytomonas* Donovan, 1909 (Fernandes et al., 1993; Flegontov et al., 2013; Lukeš et al., 2014; Maslov et al., 1996); monoxenous life cycle (involves only a single host, typically an insect, e.g. *Leptomonas* W.S.Kent, 1880, *Crithidia* L.Léger, 1902, *Herpetomonas* W.S.Kent, 1880, *Jaenimonas* Votýpka and Hamilton, 2015, *Strigomonas* (Lwoff and Lwoff, 1931) Teixeira and Camargo, 2011, *Sergeia* Svobodová et al., 2007, *Angomonas* Souza and Corte-Real, 1991, *Kentomonas* Votýpka et al., 2014, and *Zelonia* Shaw, Camargo, and Teixeira, 2016 (Barratt et al., 2017; Maslov et al., 1996; Poinar and Poinar, 2004; Svobodová et al., 2007; Teixeira et al., 2011; Votýpka et al., 2014; Wallace, 1966)).

Sexual reproduction normally has not been observed in trypanosomatids, and it is believed that most populations consist of clones (Tibayrenc and Ayala, 1999).

Trypanosomatids are notorious for causing severe diseases such as sleeping sickness (caused by *Trypanosoma brucei* Plimmer and Bradford, 1899 and transmitted by tsetse flies), Chagas disease (caused by *Trypanosoma cruzi* Chagas, 1909 and transmitted by triatomine bugs), and leishmaniasis (caused by various species of *Leishmania*). These diseases can lead to severe pathologies and have devastating effects on human health, prompting extensive research (Kaufer et al., 2017). Additionally, trypanosomes pose serious threats to domestic animals, causing diseases like nagana, surra, mal de caderas, and dourine (Büscher et al., 2019).

Of all, the classification of parasitic kinetoplastids has historically been based on the morphology of different life stages. Until 2005, only 22 genera

were described based on this approach (Simpson et al., 2006). However, molecular methods have revealed significant flaws in this taxonomy, necessitating major revisions (Maslov et al., 2013; Momen, 2001; Votýpka et al., 2015).

The genus *Trypanosoma* was first described in 1843 by David Gruby (Zídková et al., 2011). Initially, species were often classified under the "one host-one species" paradigm, suggesting strict host specificity (Podlipaev, 1990; Wallace, 1966). This led to an overestimation of species diversity among trypanosomes. It is now known that a single species can infect multiple hosts or be transmitted by several vector species. Knowledge about host-parasite relationships of avian trypanosomes remains limited (Zídková et al., 2012).

Nearly 100 species of avian trypanosomes have been described (Podlipaev, 1990), but only a few have reliable descriptions based on both morphological and molecular data (Sehgal et al., 2001; Valkiūnas et al., 2011; Votýpka and Svobodová, 2004). Historically, descriptions were based on the blood trypomastigote stage (Molyneux, 1973). The cell shape, position of the kinetoplast and the flagellum are features that help to distinguish the morphotype of trypanosome. There are eight distinguished morphotypes (Figure 1.3.2.1.) (Maslov et al., 2013). This detailed classification aids in understanding the diversity and complexity of trypanosomatid morphology and their life cycles.

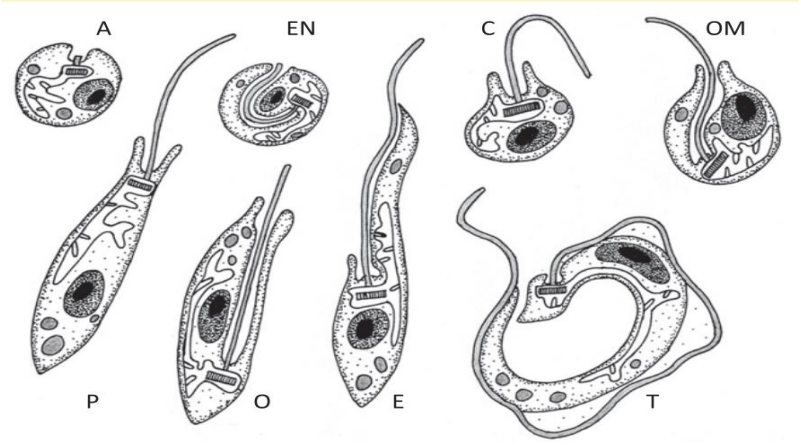

Figure 1.3.2.1. Morphotypes of trypanosomatids: A – amastigote; C – choanomastigote; E – epimastigote; EN – endomastigote; O – opisthomastigote; OM – opisthomorph; P – promastigote; T – trypomastigote (Maslov et al., 2013).

Avian trypanosomes in insects develop in the digestive tract, with species-specific locations within the tract (Bennett, 1970). Transmission to vertebrate hosts can occur through regurgitation (Volf et al., 2004), via bite site contamination with fecal matter of infected insects (Bennett, 1961), or ingestion of infected insects (Votýpka et al., 2012). Trypanosomes can inhabit the blood, bone marrow, or internal organs of vertebrate hosts, generally without causing noticeable harm, except in some cases (Bennett, 1970; Molyneux et al., 1983; Stabler et al., 1966). In contrast, they can significantly impact insect vectors by damaging the stomodeal valve, leading to blockage and destruction (Volf et al., 2004).

Monoxenous trypanosomatids are specialized parasites of Diptera species, encompassing genera such as *Leptomonas*, *Crithidia*, *Herpetomonas*, *Jaenimonas*, *Trigomonas*, *Sergeia*, *Angomonas*, *Kentomonas*, and *Zelonia* (Barratt et al., 2017; Maslov et al., 1996; Poinar and Poinar, 2004; Svobodová et al., 2007; Teixeira et al., 2011; Votýpka et al., 2004; Wallace, 1966). The discovery of *Leptomonas* and *Herpetomonas* in insects' dates back to 1851 by Burnett. Despite their long-known existence, these organisms remain neglected, primarily because they do not parasitize humans or economically significant animals. Species of genus *Crithidia*, however, is well-documented, with studies indicating no negative impact on mosquito hosts (Votýpka et al., 2001). Investigating whether monoxenous trypanosomes influence the transmission of other pathogens or affect insect hosts could offer insights into controlling populations of blood-sucking insects and parasites during outbreaks.

Dixenous trypanosomatids infect animals of all vertebrate classes, with vectors ranging from leeches to various biting flies and bugs (Fermino et al., 2015; Stevens et al., 1999). Hypotheses supported by data suggest that dixenous trypanosomes, such as *Trypanosoma*, *Leishmania*, and *Phytomonas*, evolved from monoxenous trypanosomatids (Fernandes et al., 1993; Flegontov et al., 2013; Lukeš et al., 2014; Maslov et al., 1996). Research on avian trypanosomes began in 1885 when V. Danilewsky described a flagellate from the blood of *Strix aluco* Linnaeus, 1758, naming it *Trypanosoma avium* Danilewsky, 1885 (Votýpka et al., 2002). Avian trypanosomes constitute approximately one-fifth of all described species (Podlipaev, 1990). While the life cycles of a few species are known, the vectors are not always proven but include black flies (Simuliidae), louse flies (Hippoboscidae), mosquitoes (Culicidae), biting midges (Ceratopogonidae, genus *Culicoides*), or mites (Dermanyssidae) (Baker, 1956; 1976; Desser, 1977; Miltgen and Landau, 1982; Molyneux, 1977; Mungomba et al., 1989; Votýpka and Svobodová,

2004). Experimental studies in laboratories have tested the vectorial capacity of various blood-sucking insects. Birds belonging to many orders can be parasitized by trypanosomes, with songbirds and raptors most frequently infected, while ducks, geese, and sparrows are less often infected (Apanius, 1991; Baker, 1976; Kucera, 1983). Detection methods for trypanosomes include microscopy of potential vectors' preparations, blood films of vertebrates, and the buffy coat method, PCR with specific primers, or cultivation. Depending on the method used, prevalence rates can range from below 1% to over 80% (Chagas et al., 2020; Kirkpatrick and Lauer, 1985; Kirkpatrick and Suthers, 1988; Kucera, 1983; Sehgal et al., 2001; Valkiūnas et al., 2016).

2. MATERIAL AND METHODS

2.1. Study sites

Mosquito larvae were collected in 134 water bodies in Lithuania, spanning multiple municipalities, including Vilnius and Alytus cities, Elektrėnai, Prienai, Šilutė, and Šiauliai. The coordinates for each site are detailed in Supplementary Table 1. The water bodies surveyed encompass a variety of types, such as natural wetlands, natural ponds, puddles, and vehicle ruts. Surrounding habitats included a diverse range of environments (parks, deciduous and mixed forests, meadows, urbanized areas, and abandoned areas).

Mosquito adults were collected in Vilnius: Dvarčionys (54°44'11.7"N 25°23'03.4"E), Belmontas (54°41'19.6"N 25°21'44.0"E), VU Kairėnai Botanical Garden (54°44'02.2"N 25°24'13.2"E), and Verkiai Regional Park (54°44'54.3"N 25°17'20.9"E); as well as in two other localities in the country: Puvočiai (54°06'45.9"N 24°18'08.3"E) and Brinkiškės (54°47'55.9"N 25°03'35.7"E) (Figure 2.1.1.).

Hibernating *Culex pipiens* mosquitoes were collected in damp cellars in Norkūnai village (54°30'02.5"N, 23°56'24.2"E), Panemuninkėliai village (54°25'53.0"N, 24°04'05.7"E), Palanga (55°55'05.8"N, 21°03'20.3"E), and in Vilnius (bunkers in Vilnius (54°42'01.6"N, 25°19'57.3"E), and the cellar of water tower in Verkiai Regional Park (54°44'56.9"N, 25°17'29.2"E)) (Figure 2.1.1.).

Biting midges were collected in different regions of Lithuania: Verkiai Regional Park (54°45′00″ N, 25°17′00″ E), VU Kairėnai Botanical Garden (54°44′12.5″ N, 25°24′16.4″ E), Puvočiai (54°06′52.2″ N, 24°18′17.6″ E), Ventės Ragas (55°20′28.1″ N, 21°11′25.3″ E) and its surrounding areas (55°23′57.5″ N, 21°14′14.8″ E and 55°26′12.0″ N, 21°16′04.6″ E), and Brinkiškės (54°47′55″ N, 25°03′45″ E) (Figure 2.1.1.).

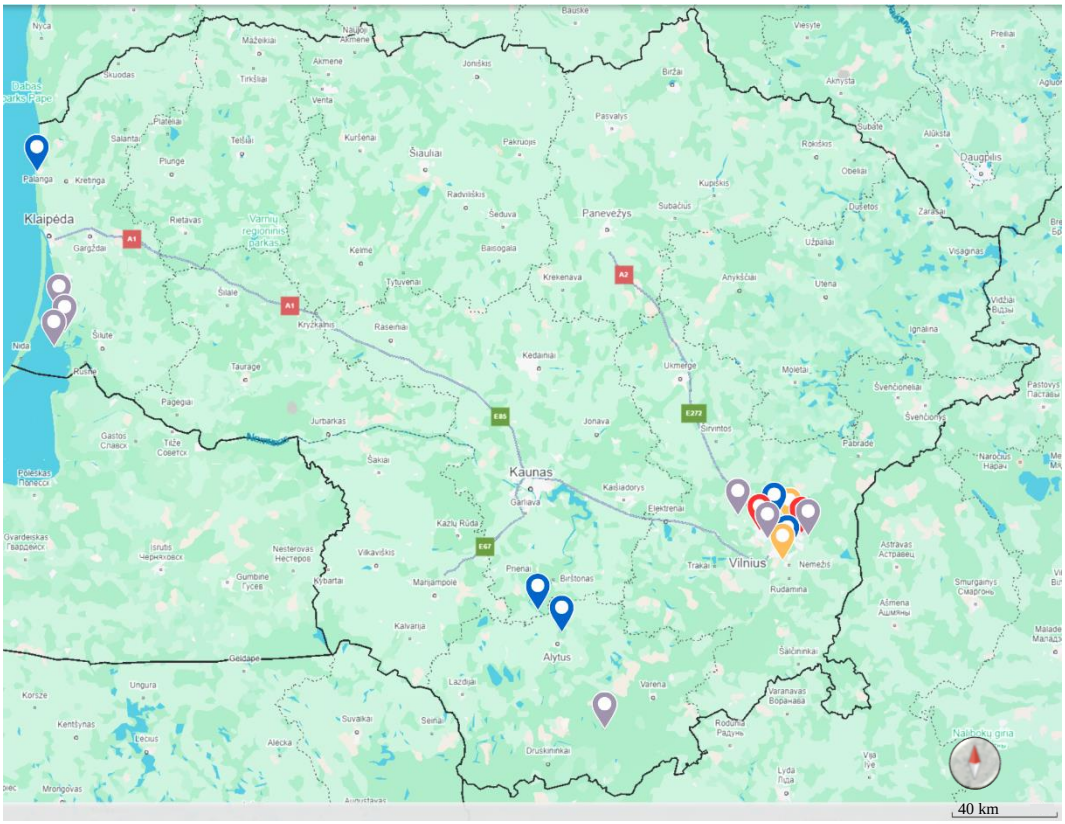

Figure 2.1.1. Map of bloodsucking insect sampling sites. Yellow symbols – the sites where sweeping net was used for mosquitoes' collection; red markers – sites where CDC traps baited with CO_2 were used for mosquitoes' collection; blue markers – hibernating sites where mosquitoes were collected using mouth aspirator; grey markers – areas where biting midges were collected using UV light traps.

2.2. Sample collection

2.2.1. Mosquito (Culicidae) larvae

Culicidae larvae were collected in 2021 (March–October) and 2022 (April–July). Depending on the location of the water body, samples were taken every two weeks or once a month. A standard dipper (⌀12,5 cm) was used for sampling. Each sampling effort consisted of three repetitions and collected mosquito larvae were placed in separate containers. The larvae were preserved in 96% ethanol until further analysis.

Water temperature (°C), pH value, and concentrations of nitrites [NO_2^- (mg/l)] and nitrates [NO_3^- (mg/l)] were measured during sampling. pH was measured using pH color-fixed indicator strips (Carl Roth, Germany), while concentrations of NO_2^- and NO_3^- were determined using Nitrate and Nitrite indicator strips (Macherey-Nagel GmbH & Co, Germany). Each water body was categorized into one of three size categories: small (less than 4 m²), medium (4–100 m²), and large (more than 100 m²). The sizes of the water bodies were measured in springtime when they were full of water.

The bottom coverage of each water body was assessed and described as being covered with sand, mud, grass, organic matter (branches, leaves, etc.), or clay. Additionally, water bodies were characterized based on their exposure to sunlight, noting whether they were in open areas with direct sunlight or in closed, shady areas. It was also recorded if the water body dried completely during the season or if it was permanent.

2.2.1. Mosquito (Culicidae) adults

Adult mosquitoes were collected in 2021–2023 (May–November). During 2021, for sampling an entomological sweeping net was used. The sampling was done every two weeks and consisted of three repetitions for five minutes. Collected mosquito adults were placed in containers with 96% ethanol for further transportation and storing until identification.

In 2022 and 2023 – CDC traps baited with CO_2 were used. Traps were hung 1.5–2 m above the ground and opened 6–7 h before sunset and taken down 4–5 h after sunrise, and material was taken to the lab for further analysis right after.

Hibernating *Culex pipiens* mosquitoes were collected in December–January, 2023–2024. Flashlights were used to locate mosquitoes, and mouth aspirators were used for collection. The material was transported to the laboratory on the same day for further processing.

2.2.2. Biting midge (*Culicoides*) adults

Biting midge adults were collected in 2021–2022 (June–September). UV LED-light traps (BG-Pro All-In-One Biogents AG) were used for these insects sampling. Traps were placed 1.5–2 m above the ground at the same time of day as mosquito traps. Biting midges were collected to small cups with water and a drop of liquid soap. The collected material was transported to the lab for further analysis.

2.3. Insect identification and dissection

For insect species identification, 4th instar mosquito larvae were separated; this stage is used for identification, because all of the features are fully developed. Identification was performed using the morphological keys provided in Becker et al. (2003) and the MosKeyTool interactive identification tool (Gunay et al., 2018). The larvae were examined under a stereo microscope (MOTIC SMZ-171, China), without performing dissections.

Initially, the collected adult mosquitoes were inactivated by placing them in the freezer for 10–15 minutes. The mosquitoes were then sexed and sorted. Female mosquitoes were identified morphologically under a stereo microscope (MOTIC SMZ-171, China) using the identification keys by Becker et al. (2003) and the MosKeyTool interactive database (Gunay et al., 2018). For the mosquitoes collected in 2021, females were dissected to separate the abdomens for molecular analysis. Samples were processed individually or in pools (two to five mosquitoes per pool) consisting of abdomens from the same mosquito species collected at the same location and on the same day. This material was stored in SET buffer for DNA extraction and PCR-based detection of parasites.

Mosquito adult females collected in 2022–2023 were processed individually. Each mosquito was placed on a clean microscope glass slide with a drop of 0.9% saline solution. The head was detached from the thorax, which was then gently pressed to extract the contents of the anterior part of the thorax as salivary glands of mosquitoes are located in this part. Salivary glands are visible under a stereo microscope. They were isolated, gently crushed, spread thinly on the slide, and dried at room temperature. The remnants of the dissected mosquito was transferred to a clean slide, where the last two abdominal segments were carefully pulled along with the digestive tract. The midgut was separated from the other organs, spread thinly on a slide, and left to dry at room temperature. To prevent contamination, all dissecting needles were sterilized with fire after each dissection. The dried slides containing salivary gland and midgut preparations were then fixed with a drop of absolute methanol and stained with a 4% Giemsa solution for one hour (Valkiūnas, 2005; Žiegytė et al., 2017). This process allows the visualization of sporozoites (the infective stage of *Plasmodium* parasites) in the salivary glands, confirming the completion of parasite development in the mosquito. The preparations of biting midge midguts allow the detection of

trypanosomatids. The remaining parts of the mosquitoes were individually preserved in SET buffer for further PCR-based analysis.

Hibernating *Culex pipiens* mosquitoes (collected in 2023–2024) were dissected for salivary gland and midgut preparations and insect remnants were pooled in groups of 10 mosquitoes per pool to screen for the presence of haemosporidian parasites and trypanosomatids using PCR. Positive samples were then examined microscopically.

Biting midges were sexed, and those with a burgundy pigment were selected. The pigment presence is an indication of at least one gonotrophic cycle (Dyce, 1969), suggesting the midges had consumed at least one blood meal and were more likely to harbor sporozoites of haemosporidian parasites. Each insect was then individually placed on a drop of 0.9% saline solution on a clean microscope slide. The head and wings were removed and transferred to a new slide containing a small drop of Euparal, covered with a cover slide, and left to dry either at room temperature for two months or in an incubator at 60°C for one week. These permanent preparations were later used for the morphological identification of the dissected *Culicoides* with Glukhova et al. (1989), Gutsevich (1973), and Mathieu et al. (2012) keys for morfological identification.

The salivary glands, located in the anterior portion of the insect's thorax (Valkiūnas, 2005), were carefully crushed with dissecting needles to create a small, thin smear (Žiegytė et al., 2017). To prevent contamination, all dissecting needles were sterilized with fire after each dissection. The salivary gland preparations were air-dried, fixed with a drop of absolute methanol, and stained with a 4% Giemsa solution (Valkiūnas, 2005; Žiegytė et al., 2017). The remnants of the dissected biting midges were preserved in 96% alcohol for PCR-based analysis.

All salivary gland, midgut, and biting midge head and wings permanent preparations are stored at the Nature Research Centre, Vilnius, Lithuania.

2.4. Analysis of microscopical preparations

Both slides of mosquito and biting midges which were determined to be PCR positive for investigated parasites (see chapter 2.5.) were examined using Olympus BX-43 light microscope equipped with an Olympus DP12 digital camera and the image software Olympus DP-SOFT (Olympus, Japan). In cases of positive *Plasmodium* samples in mosquitoes and *Haemoproteus* samples in biting midges, salivary gland preparations were screened for presence of infective stages – sporozoites. In cases of positive samples for

trypanosomatids, preparations of mosquito salivary gland and midgut preparations were analyzed the same way. The width, length, and area of sporozoites were measured using ImageJ v.1.54g software (Schneider et al., 2012). For both haemosporidian parasites and trypanosomatids the samples were examined entirely under ×1000 magnification. Representative preparations of sporozoites were deposited at the Nature Research Centre, Vilnius, Lithuania (sporozoites in biting midge salivary glands: 49409NS–49425NS; NS49742–NS49757; sporozoites in mosquito salivary glands: 49758NS; trypanosomes in mosquito preparations: 49805NS–49807NS).

2.5. PCR-based analysis

2.5.1. DNA extraction

Samples (pooled mosquito abdomens) collected in 2021, pools of hibernating *Culex pipiens* females (collected in 2023–2024), and all investigated biting midges after dissections had their total DNA extracted using an ammonium acetate protocol (Richardson et al., 2001). DNA from samples (mosquitoes after dissection) collected in 2022–2023 was extracted using the GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Lithuania) following the manufacturer's instructions.

2.5.2. Polymerase chain reactions

Screening for haemosporidian parasites (*Plasmodium* and *Haemoproteus* species):

For detecting haemosporidian parasite DNA in mosquitoes and biting midges, a nested PCR protocol (Bensch et al., 2000; Hellgren et al., 2004) with two pairs of primers was used to amplify a 479 bp length fragment of the cytochrome *b* gene (*cytb*):

1. First PCR: Primers HaemNFI (5′-CATATATTAAGAGAAITATGGAG-3′) and HaemNR3 (5′-ATAGAAAGATAAGAAATACCATTC-3′) amplify a fragment of *Haemoproteus*, *Leucocytozoon*, and *Plasmodium*.
2. Nested PCR: Inner primers HaemF (5′-ATGGTGCTTTCGATATATGCATG-3′) and HaemR2 (5′-GCATTATCTGGATGTGATAATGGT-3′) are specific to *Haemoproteus* and *Plasmodium* genera.

All PCR reactions were performed in a total volume of 25 µL, which included 2 µL of genomic DNA template, 12.5 µL of DreamTaq Master Mix

(Thermo Fisher Scientific, Lithuania), 8.5 µL of nuclease-free water, and 1 µL of each primer. The temperature cycles adhered to the original protocol: the initial PCR started with denaturation at 94°C for 3 minutes, followed by 20 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds, concluding with a final extension at 72°C for 10 minutes. For the second amplification the same conditions were used, except instead of 20 cycles, 35 cycles were used. For each amplification, negative control (nuclease-free water) and positive control (samples of infected blood confirmed by microscopy) were used.

Multiplex PCR for haemosporidian parasites:

During the search for haemosporidian parasites in mosquitoes some PCR-positive samples presented several double peaks, indicating possible haemosporidian co-infections. In these cases, a multiplex PCR protocol was conducted as described by Ciloglu et al. (2019). This protocol amplifies fragments of different sizes of the mitochondrial DNA of *Plasmodium* (377–379 bp) (primers used: PMF (5'- CCTCACGAGTCGATCAGG-3'), PMR (5'- GGAAACCGGCGCTAC-3')), *Haemoproteus* (525–533 bp) (primers used: HMF (5'- ATTGGATGTCAATTACCACAATC-3'), HMR (5'- GGGAAGTTTATCCAGGAAGTT-3')), and *Leucocytozoon* (218 bp) (primers used: LMF (5'- TGGAACAATAATTGSATTATTTACAYT-3'), LMR (5'- AACATATCATATTCCATCCATTTAGATTA-3')) simultaneously, allowing the PCR product to be visualized in the electrophoresis gel for genus identification without sequencing. PCR reactions were carried out in a total volume of 27 µL, for that 12.5 µL 2X Phusion Master Mix with GC Buffer (Thermo Fisher Scientific, Lithuania) was used, together with 6.5 µL of nuclease-free water, 1 µL of each primer, and 2 µL of total genomic DNA template. PCR thermal regiment was initial denaturation of 98°C for 30 seconds, after those 35 cycles of 98°C for 10 seconds, 59°C for 45 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 10 minutes.

Screening for trypanosomatida:

For trypanosomatid detection in *Culex pipiens* mosquitoes, a nested PCR protocol (Sehgal et al., 2015; Valkiūnas et al., 2011) was employed using two pairs of primers to amplify a DNA fragment encoding 18S rRNA of 750 bp length:

1. First PCR: Primers Tryp763 (5'- CATATGCTTGTTTCAAGGAC-3') and Tryp1016 (5'- CCCCATAATCTCCAATGGAC-3').

2. Nested PCR: Inner primers Tryp99 (5'-TCAATCAGACGTAATCTGCC-3') and Tryp957 (5'-CTGCTCCTTTGT TATCCCAT-3').

All PCR reactions were carried out in a total volume of 25 µL, consisting of 2 µL of total genomic DNA template, 12.5 µL of DreamTaq Master Mix (Thermo Fisher Scientific, Lithuania), 8.5 µL of nuclease-free water, and 1 µL of each primer. The temperature profiles for all PCRs followed the original protocol: the first PCR began with denaturation at 95°C for 5 minutes, followed by 5 cycles of 95°C for 60 seconds, 45°C for 30 seconds, and 65°C for 60 seconds, then 35 cycles of 95°C for 60 seconds, 50°C for 30 seconds, and 72°C for 60 seconds, with a final extension at 65°C for 10 minutes. The second PCR involved denaturation at 96°C for 3 minutes, followed by 25 cycles of 96°C for 30 seconds, 58°C for 60 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 7 minutes.

***Culicoides* species identification using PCR:**

To confirm some biting midge's species or identify PCR-positive females from the *Culicoides obsoletus* group, the primers LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') were used to amplify a fragment of about 600 bp of the cytochrome c oxidase subunit I (COI) of the mitochondrial DNA of insects (Folmer et al., 1994). Original protocol is modified: same as with hemosporidian and trypanosomatid PCRs reactions were performed in a total volume of 25 µL, which included 2 µL of genomic DNA template, 12.5 µL of DreamTaq Master Mix (Thermo Fisher Scientific, Lithuania), 8.5 µL of nuclease-free water, and 1 µL of each primer. The temperature cycles were the same as in the original protocol: 35 cycles at 95°C for 60 seconds, 40°C for 60 seconds, and 72°C for 90 seconds, with a final extension at 72°C for 7 minutes.

***Culex pipiens* and *Culex torrentium* distinguishing using restriction enzymes:**

Culex pipiens and *C. torrentium* females can only be distinguished by molecular methods. For this purpose, the applied method is based on the use of restriction enzymes (SspI and/or FspBI) to separate these two species as described by Hesson et al. (2010). First, the COI region of mitochondrial DNA was amplified using primers C1-J-2183 (5'-CAACATTTATTTTGATTTTTTGG-3') and TL2-N-3014 (5'-TCCATTGCACTAATCTGCCATATTA-3') (Simon et al., 1994). The amounts of reagents are the same as in previously descripted PCR reactions. Thermocycler conditions: initial denaturation at 95°C for 3 minutes, then 30

cycles of 94°C for 30 seconds, 49.8°C for 30 seconds, and 72°C for 1 minute, ending with extension of 72°C for 7 minutes. After this PCR a digestion was carried out in 30 µL reactions, consisting of 17 µL of nuclease-free water, 2 µL of 10X FastDigest Buffer, 10 µL PCR product, and 1 µL of FastDigest enzyme (either SspI or FspBI). Then tubes are incubated at 37°C for 5 minutes after which the results are visualized in 2% agarose gel stained with MidoriGreen dye (NIPPON Genetics Europe, Germany). SspI enzyme cuts a fragment of *C. pipiens* into two (~620 pb and 210 bp) and the PCR product of *C. torrentium* remains uncut. With enzyme FspBI it's the opposite of previously described one. This method enables distinction of two sibling species *C. pipiens* and *C. torrentium* without sequencing.

2.6. Electrophoresis

PCR products were visualized in 2% agarose gel using MidoriGreen dye (NIPPON Genetics Europe, Germany). Samples containing a band of ~500 bp for haemosporidian parasites, ~750 bp for trypanosomatids, and ~600 bp for insects were considered positive. In cases of searching for haemosporidian parasites and trypanosomes nested PCR-positive products were purified using an ammonium acetate protocol (Sambrook and Green, 2012) and sequenced in both strands with inner primers using an Applied Biosystems Genetic Analyzer 3500. Same was done with insect samples.

Multiplex PCRs' results were also visualized on 2% agarose gel, that was stained with MidoriGreen (NIPPON Genetics Europe, Germany). Bands corresponding with the lengths of previously given show presence of *Plasmodium, Haemoproteus,* and *Leucocytozoon* parasites. Positive samples for multiplex PCR were not sequenced as the goal was to evaluate if multiple infections were caused by different haemosporidian genera and to determine which ones. On the other hand – the DNA segments amplified using this protocol are not typically used for barcoding.

2.7. Sequence analysis

All chromatograms of the obtained sequences were analyzed, and contig sequences (when both strands of a sample were sequenced) were assembled using Geneious Prime 2023.2.1 (https://www.geneious.com) and BioEdit 7.7.1. To detect haemosporidian species and lineages, the BLAST function in the MalAvi database (http://mbio-serv2.mbioekol.lu.se/Malavi) was used, and results were cross-checked with the "Basic Local Alignment Search Tool" on the National Center for Biotechnology Information (NCBI) website

(http://www.ncbi.nlm.nih.gov/BLAST) as well as “Identification Engine” function in BOLD system (https://www.boldsystems.org/index.php/IDS_OpenIdEngine). Matches with 100% similarity were considered positive for known haemosporidian lineages, while sequences with at least one base-pair difference from previously deposited sequences were classified as new lineages (Bensch et al., 2009). For positive samples showing new connections (previously not recorded haemosporidian lineages in certain mosquito species) parasite lineages were deposited in GenBank (Table 2.7.1.). For biting midges, all obtained sequences were deposited in GenBank (Table 2.7.1.). The BLAST tool on the NCBI website and BOLD system were used to identify trypanosomatids and insect DNA. Parasite sequences obtained from trypanosomatid-positive samples were deposited in GenBank (Table 2.7.1.).

Biting midge species identifications were done for DNA sequences presenting > 99% similarity. Morphological identification was consistent with PCR-based identification of the insects in all cases. These sequences were deposited in GenBank (Table 2.7.1.).

A phylogenetic tree for trypanosomatid sequences was created using Geneious Prime 2023.0.4 software. The best-fit model for phylogenetic analysis (GTR+I+G) was determined using jModeltest-2.1.10 software. The tree was constructed with the MrBayes plugin v3.2.6, running for 6 million generations with sampling every 100th generation. The first 25% of trees were discarded as a 'burn-in' phase before constructing the final consensus tree.

Table 2.7.1. GenBank accession numbers of sequences that were deposited during the present research.

	Found in mosquitoes	**Found in biting midges**
Haemosporida	PP755166–PP755176	OP546062–OP546095
Trypanosomatida	PP946099–PP946107; PP94731, PP948732	–
Insect species	–	OP692758–OP692766

2.8. Statistical analysis

Mosquito larvae: The average number of mosquito larvae per sample (one water body during one sampling) and the standard error (SE) were calculated using Microsoft Excel 365. For 12 mosquito species, that occurred in more than five different samples, multiple regression analysis was applied

using STATISTICA 12.5 software to determine which parameters were related to the abundance of mosquito larvae in different water bodies. Multivariate redundancy analysis (RDA) was applied to find associations between the abundance of mosquito species and quantitative variables using Brodgar 2.7.5. software (Highland Statistics Ltd.).

The density (D) of mosquito larvae species was calculated as a proportion of certain mosquito species to the total number of mosquitoes (given as %). Mosquito distribution (C) was calculated as the percentage (%) of water bodies where a certain species was found in comparison to all investigated water bodies (Bocková et al., 2013).

Mosquito adults: The mean and standard deviation (SD) of the width, length, and area of sporozoites were calculated using Microsoft Excel 365. To evaluate overall mosquito infection, the minimum infection rate (MIR) was calculated as provided by Schoener et al. (2017). When analyzing pooled mosquitoes, if a pool was positive for haemosporidian infection, it was assumed that one mosquito from the pool was infected:

$$MIR(\%) = n_{(\text{positive samples})} / N_{(\text{all mosquitoes})} \times 100$$

Chi-square test for independence was performed to compare the prevalence of haemosporidian parasites in different mosquito species. Comparison of hibernating and active *Culex pipiens* mosquitoes was performed by applying Fischer's exact test. For both tests *P* value of 0.05 or less was considered significant.

Biting midges (biting midges species and haemosporidian lineages Interaction Analysis): To understand the interactions between biting midges and their *Haemoproteus* parasites in Europe, studies published on the continent to date were used. These studies were accessed using the PubMed and Google Scholar databases with the search terms: "*Culicoides* AND Haemosporida," "*Culicoides* AND Haemosporidian," "*Culicoides* AND *Haemoproteus*," and "*Haemoproteus* AND vectors." Only studies that used molecular methods and amplified a fragment of the *cytb* gene were included in this analysis. For studies that analyzed samples using insect pools, each pool was considered one sample. Information for each *Culicoides* species containing the same *Haemoproteus cytb* lineage was summarized to obtain an interaction matrix, showing the frequency of interaction consisting of the number of infected biting midges by a particular parasite lineage. A bipartite network and an adjacency matrix organized in modules of *Haemoproteus*

lineages and *Culicoides* spp. were constructed in R 4.0.5 using the bipartite package.

For comparison of prevalence of haemosporidian parasites in different biting midge species Chi-square test for independence was performed.

3. RESULTS AND DISCUSSION

3.1. The diversity of mosquito larvae, seasonality, and certain physical and chemical parameters that affects abundance of mosquito larvae

3.1.1. Results

A total of 606 samples were collected, with 225 testing positive for mosquito larvae. Each water body was surveyed between one to ten times during the season, depending on whether it dried up or reappeared during the study period. In total, 134 water bodies were examined for mosquito larvae, with 101 found to contain larvae at least once. Mosquito larvae were observed from March 31st, when the water temperature was 7°C, until September 30th, when it was 9°C. Of the 5392 mosquito larvae collected, 2141 were 4th instar larvae (154 out of 225 samples) and were identified to the species level. Twenty-five mosquito species were identified, belonging to five genera (Table 3.1.1.1.). The most numerous were species of the genus *Ochlerotatus*, comprising 67.6% of all collected mosquitoes, followed by *Aedes* at 25.2%, while *Culiseta*, *Culex*, and *Anopheles* species constituted 3.4%, 3.3%, and 0.5%, respectively. On average, 8.9±1.5 mosquito larvae were captured per sample, with the maximum number being 381 in a single sample in early April. In April 2021, the majority (50.6%) of mosquito larvae were collected, despite the consistent sampling method throughout the season. The number of larvae was also high in May (28.4%) but dropped to 2.8% in June for both years. The first appearance of mosquito larvae was in March in both 2021 and 2022 (Figure 3.1.1.1.).

In 99 of the samples positive for mosquito larvae (64.3%), only one mosquito species was identified. *Anopheles maculipennis* and *Ochlerotatus riparius* were the only species found exclusively without any other species, while other species were found either alone or coexisting with other species.

In 44 samples (28.6%), two species were present simultaneously (Table 3.1.1.2.). In nine samples (5.8%), three mosquito species were detected, but all three were found together only once (Table 3.1.1.2.). In two instances, four species were found in a single sample: *Aedes cinereus* was found with *Culex pipiens*, *Culiseta annulata*, and *Culiseta morsitans*; *Ochlerotatus annulipes* was found together with *Ochlerotatus cantans*, *Ochlerotatus cataphylla*, and *Ochlerotatus punctor*.

Table 3.1.1.1. Mosquito species identified in investigated water bodies with the number (n) of water bodies where larvae of certain species have been found, distribution (C), number (L) of individuals of certain species found throughout the season and density (D).

Mosquito species	**n**	**C (%)**	**L**	**D (%)**
Aedes cinereus	47	35.1	367	17.1
A. vexans	6	4.48	172	8.03
Anopheles claviger	2	1.49	2	0.09
A. maculipennis	2	1.49	8	0.37
Culiseta alaskaensis	4	2.99	16	0.75
C. annulata	6	4.48	42	1.96
C. morsitans	11	8.21	16	0.75
Culex pipiens	5	3.73	47	2.2
C. territans	6	4.48	9	0.42
C. torrentium	1	0.75	14	0.65
Ochlerotatus annulipes	5	4.48	41	1.91
O. behningi	1	0.75	2	0.09
O. cantans	48	35.8	607	28.4
O. caspius	2	1.49	6	0.28
O. cataphylla	14	10.5	274	12.8
O. communis	3	2.24	30	1.4
O. diantaeus	1	0.75	4	0.19
O. euedes	2	1.49	30	1.4
O. flavescens	13	9.7	53	2.48
O. geniculatus	1	0.75	2	0.09
O. intrudens	2	1.49	4	0.19
O. nigrinus	2	1.49	12	0.56
O. punctor	11	8.21	29	1.35
O. riparius	1	0.75	6	0.28
O. sticticus	5	3.73	348	16.3

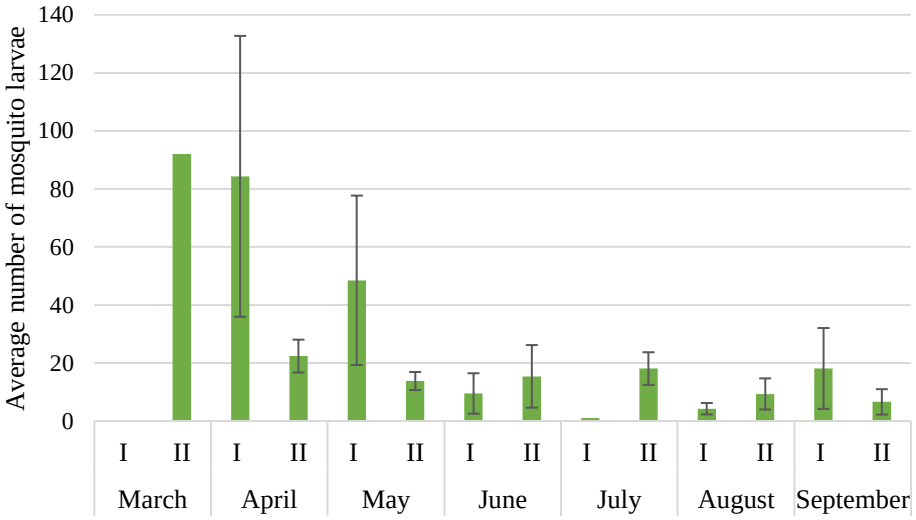

Figure 3.1.1.1. Average number of mosquito larvae caught per sampling each half of the month in 2021 (Average and SE values; SE is not calculated for March since only in one sample mosquito larvae were found).

pH value was 7.5 in 35.2% of all tested water bodies, but overall, it varied between 5.5 and 8 (Figure 1.3.1.2.). NO_2^- concentrations ranged from 0 to 0.5 mg/L, while NO_3^- concentrations were between 0 and 50 mg/L, with more than 91% of water bodies having no detectable nitrites or nitrates.

The bottoms of the investigated water bodies were covered with various types of organic matter: 31.3% had a mix of organic materials, 28.1% were covered exclusively with tree leaves, 20.3% with grass, 17.2% with mud, and 3.1% with sand. More than half (52.3%) of the surveyed water bodies were of medium size, a quarter (25.0%) were large, and the remaining (22.7%) were small. Throughout the season, 58.6% of the water bodies dried up completely at some point, whereas 41.4% retained water throughout. Additionally, 64.7% of the water bodies were in the shade, while the remaining 35.3% were exposed to sunlight for at least part of the day (Figure 3.1.1.3.).

Multiple regression analysis indicated that the abundance of *Aedes* mosquitoes was significantly higher in water bodies with bottoms covered by leaves ($p=0.000$, $\beta=0.340$). *Anopheles* mosquito abundance was associated with the collection time ($p=0.026$, $\beta=0.098$), with these mosquitoes being primarily collected in June and September 2021. *Culex* mosquitoes were predominantly found in temporary water bodies ($p=0.041$, $\beta=0.090$). The abundance of *Culiseta* mosquitoes was related to both the collection time ($p=0.019$, $\beta=0.103$) and the drying up of the water body ($p=0.025$, $\beta=0.097$),

with 85.7% of *Culiseta* mosquitoes found in temporary water bodies. *Ochlerotatus* abundance did not show any significant relationships with the investigated parameters.

Table 3.1.1.2. Mosquito species that were found together in the same samples. The top right of the table shows samples where two species were found together (the number indicates how many samples of two species were found together). The bottom left part of the table shows cases where 3 species were found together; the third species is indicated by the letter: a – *Culiseta alaskaensis*, b – *Culex pipiens*, c – *O. annulipes*, d – *O. cantans*, e – *O. caspius*, f – *O. diantaeus*, g – *O. flavescens*, h – *O. intrudens*.

Mosquito species	*Aedes cinereus*	*A. vexans*	*A. claviger*	*Culiseta annulata*	*C. morsitans*	*Culex territans*	*C. torrentium*	*O. annulipes*	*O. behningi*	*O. cantans*	*O. cataphylla*	*O. communis*	*O. euedes*	*O. flavescens*	*O. geniculatus*	*O. intrudens*	*O. nigrinus*	*O. punctor*	*O. sticticus*
A. cinereus		1		1					1	7				5			1		2
A. vexans										1	1								1
A. claviger																			
C. annulata	+d																		
C. morsitans								1		1									
C. territans								1											
C. torrentium			+a																
O. annulipes											1								
O. behningi																			
O. cantans	+b										7	1	1				1	6	
O. cataphylla	+d				+c					+f				1					1
O. communis											+g								
O. euedes																			
O. flavescens																			
O. geniculatus																		1	
O. intrudens																			
O. nigrinus																			
O. punctor										+h						+e			
O. sticticus																			

The multiple regression analysis revealed that certain mosquito species exhibit seasonal dependence. For instance, *Culex territans* was found in August 2021, *O. cantans* was mostly found in April during both years, and *O. punctor* was also found in April (Tables 3.1.1.3.; 3.1.1.4.). Larvae of *Aedes cinereus* and *Culiseta annulata* were significantly more abundant in temporary water bodies (Figure 3.1.1.3., Table 3.1.1.3.). The amount of nitrates in the water negatively affected the abundance of *Culex pipiens* larvae while

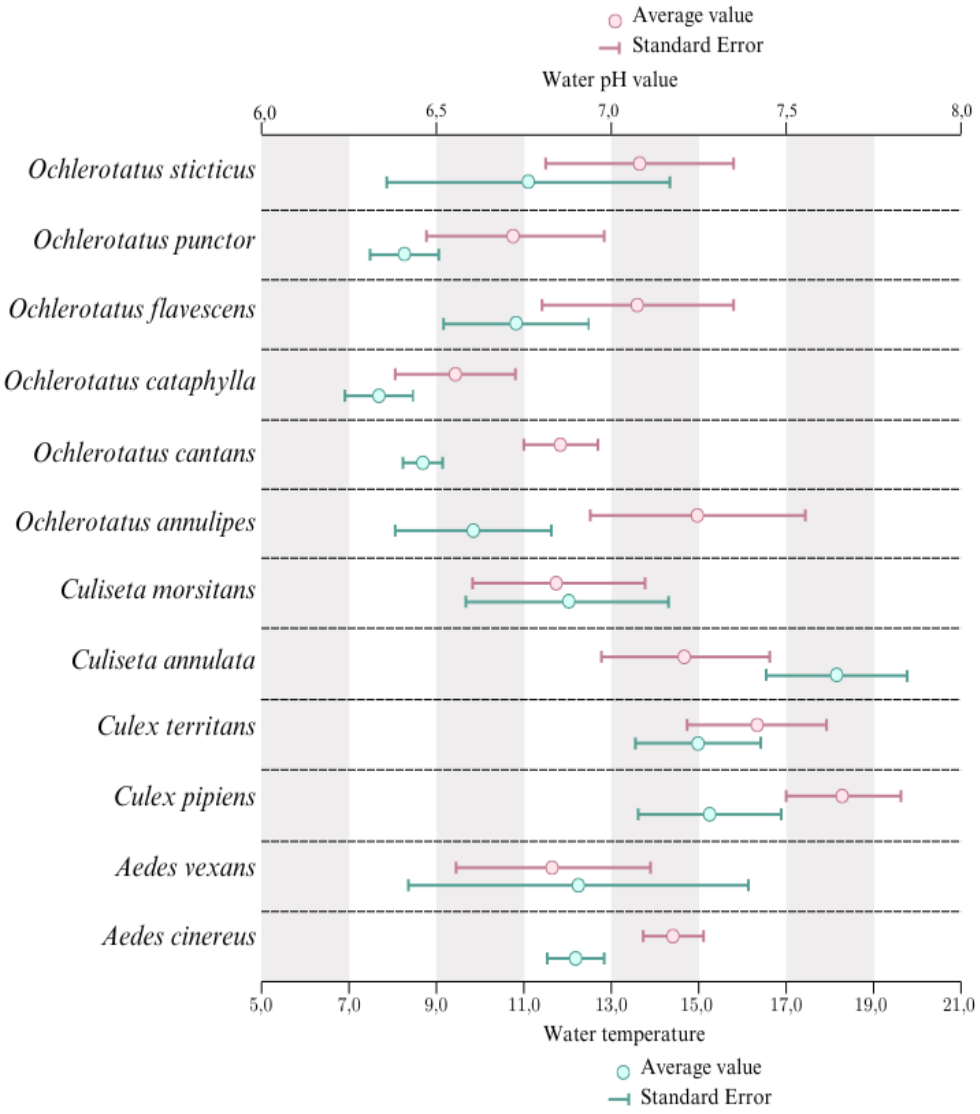

Figure 3.1.1.2. Average water temperatures and pH values with standard error values measured in water bodies in which the most abundant mosquito species were found.

positively influencing the abundance of *O. cantans* larvae (Table 3.1.1.3.). *Ochlerotatus cantans* abundance was related to specific pH levels (Figure

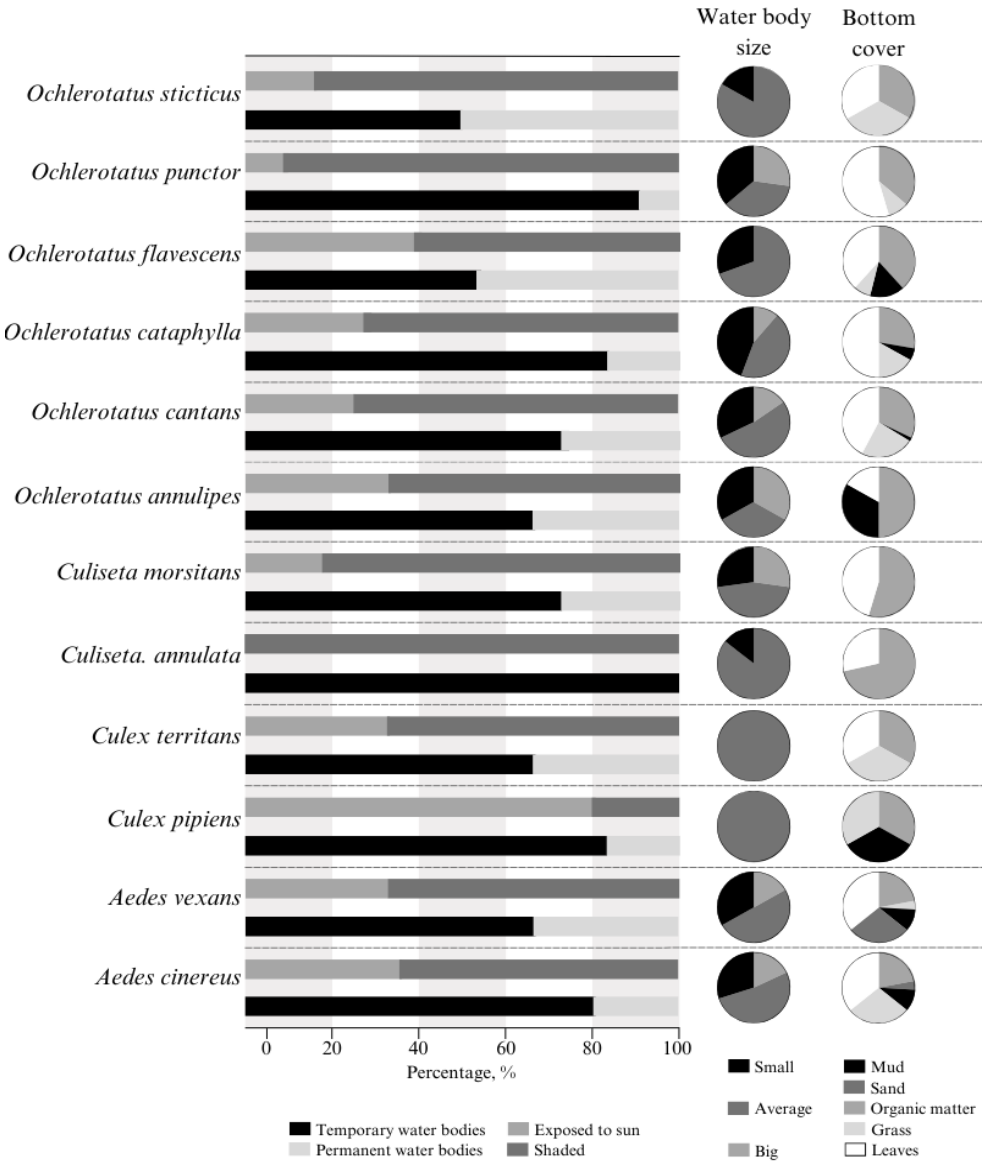

Figure 3.1.1.3. Data on each species preference for temporary or permanent water bodies, open or shaded water bodies, size and bottom cover of water bodies as observed in this research.

1.3.1.2., Table 3.1.1.3.), and *C. annulata* larvae were mostly collected in water bodies covered by organic matter (Figure 3.1.1.3., Table 3.1.1.3.).

Table 3.1.1.3. Multiple regression analysis data showing the significant correlations between most abundant species and the prevalent water parameters of the collecting sites.

		b*	Std.Err of b*	b	Std.Err of b	t (591)	p-value
Aedes cinereus	Permanent/ temporary water bodies	0.09	0.04	0.63	0.32	2.00	≤0.05
Culex pipiens	NO_3^-	0.11	0.04	0.04	0.02	2.70	≤0.01
C. territans	Sampling time	0.12	0.04	0.01	0.00	2.63	≤0.01
Culiseta annulata	Permanent/ temporary water bodies	0.11	0.04	0.18	0.07	2.48	≤0.01
	Bottom cover	0.11	0.04	0.05	0.02	2.60	≤0.01
Ochlerotatus cantans	Sampling time	-0.11	0.04	-0.44	0.18	-2.46	≤0.01
	pH	-0.09	0.04	-0.91	0.46	-1.98	≤0.05
	NO_3^-	0.11	0.04	0.26	0.09	2.83	≤0.00
O. cataphylla	Sampling time	-0.09	0.04	-0.16	0.08	-1.95	≤0.05
O. punctor	Sampling time	-0.10	0.04	-0.03	0.01	-2.34	≤0.02

b – Unstandardized Regression Coefficient
b* – Standardized Regression Coefficient

Redundancy analysis confirmed the results obtained using multiple regression, indicating that *O. cantans*, *Aedes vexans*, and *A. cinereus* were more abundant in water bodies with higher concentrations of NO_2^- and NO_3^- (Figure 3.1.1.4.). The analysis also revealed positive relationships between the abundance of *Culiseta annulata*, *Culex pipiens*, and *Culex territans* and factors such as pH value, water temperature, and collection time. These mosquito species were more abundant in the latter part of the summer when water temperatures were higher. Conversely, the abundance of *O. punctor*, *O. flavescens*, and *O. annulipes* was greater in colder water, and the abundance

of *O. cataphylla* was negatively related to pH values, as this species was found in water bodies with the lowest pH levels (Figures 3.1.1.2.; 3.1.1.4.).

Table 3.1.1.4. The percentage of 12 the most abundant mosquito species during the 2021 season each month (the percentage per month is calculated from the total number of mosquito larvae individuals of certain species).

Species	**Months when the species were present (%)**					
	April	May	June	July	August	September
Aedes cinereus	6.6	57.2	11.2	0	23.4	1.6
A. vexans	5.8	29.7	0	64.5	0	0
Culiseta annulata	0	0	57.1	19.1	23.8	0
C. morsitans	37.5	0	6.3	12.5	18.7	25.0
Culex pipiens	0	0	6.4	27.7	63.8	2.1
C. territans	0	0	0	22.2	55.6	22.2
Ochlerotatus cantans	90.1	7.0	1.9	0	0.8	0.2
O. annulipes	66.7	33.3	0	0	0	0
O. cataphylla	75.3	24.7	0	0	0	0
O. flavescens	52.8	35.8	11.4	0	0	0
O. punctor	100	0	0	0	0	0
O. sticticus	23.6	22.1	0	54.3	0	0

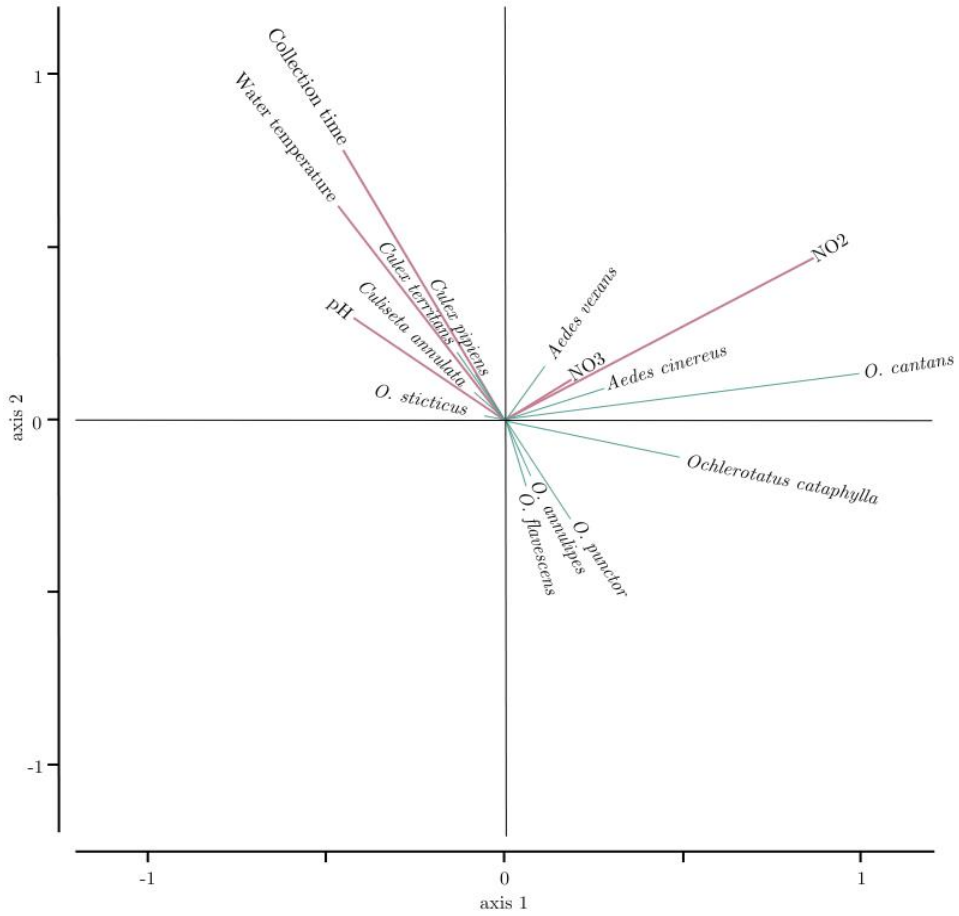

Figure 3.1.1.4. Redundancy analysis showing mosquito species relationships with investigated parameters. The two-dimensional approximation explained 94.3% of this (76.9% on axis 1 and 17.4% on axis 2).

3.1.2. Discussion

During this research, 25 mosquito species were found, making up 69.4% of all Culicidae species reported in Lithuania (Bernotienė and Lučiūnaitė, 2011; Pakalniškis et al., 2006). Podėnaitė (1959; 1962) investigated mosquito larvae in various regions of Lithuania and found larvae of 26 species in 1959 and 22 species in 1962 (Table 3.1.2.1.).

Further surveillance and long-term monitoring of mosquito larvae across more diverse habitats might increase the number of mosquito species identified in Lithuania.

Table 3.1.2.1. Comparison of species that were found in the current study with the results obtained by Podėnaitė in 1959 and 1962.

	Podėnaitė, 1959	Podėnaitė, 1962	Current research
Aedes cinereus	+	+	+
A. vexans	+	+	+
Anopheles claviger	+		+
A. maculipennis	+	+	+
Culex pipiens	+	+	+
C. territans			+
C. torrentium			+
C. apicalis	+		
Culiseta alaskaensis		+	+
C. annulata	+	+	+
C. morsitans	+		+
Ochlerotatus annulipes	+	+	
O. behningi	+		+
O. cantans	+	+	+
O. caspius	+	+	+
O. cataphylla	+	+	+
O. cyprius	+	+	
O. communis	+	+	+
O. diantaeus	+	+	+
O. dorsalis	+	+	
O. euedes	+	+	+
O. excrucians	+	+	
O. flavescens	+	+	+
O. geniculatus	+		+
O. intrudens	+	+	+
O. leucomelas	+	+	
O. nigrinus		+	+
O. pullatus	+		
O. punctor	+	+	+
O. riparius	+	+	+
O. sticticus	+		+

We gained knowledge on the seasonality of mosquitoes at our study sites. Data shows that the highest abundance of mosquito larvae in water bodies occurs from April through May, but larvae of different species can be found from the end of March until the end of September. Mosquito species belonging to the genus *Aedes* had two peaks during the season, genera *Culiseta* and *Culex* are more active in the second part of summer, although some *Culiseta morsitans* larvae were collected in April, and genus *Ochlerotatus* can be found

in spring and early summer, but *O. sticticus* seems to have two generations, with the second more numerous (54.3%) in July (Table 3.1.1.4.).

We determine relationships between certain parameters and the presence and abundance of mosquito larvae of different species in the investigated water bodies. Statistical analysis showed that collection time, temporality of the water bodies, the amount of nitrates in water, pH levels, and bottom coverage impact the presence of mosquito larvae of some species, but the abundance of neither species was associated with exposure to the sun or water body size.

Some mosquito species were found in a wide range of investigated parameters: for example, *Aedes vexans*, *O. annulipes*, *O. flavescens*, and *O. sticticus* (both water temperature and pH values) (Figure 3.1.1.2.). In contrast, other species were found in a narrow range, such as *O. cantans*, *O. cataphylla*, and *Aedes cinereus* (Figure 3.1.1.2.). Interestingly, according to Becker et al. (2003), mosquito larvae of these latter three species can often be found developing together.

Certain mosquito species' abundance had a statistically significant relationship with investigated parameters: for example, *O. cantans* showed a narrow range of water temperature and pH values (Figure 3.1.1.2.). Interestingly, *O. cataphylla* and *O. cantans* were commonly found together (15.9% of species pairs). Becker et al. (2003) noted that *O. cataphylla* develops more abundantly in neutral and slightly alkaline water, but our data showed that larvae of this species were found in slightly acidic (pH = 6) waters. We found larvae of *O. cantans* in water bodies with pH values averaging 6.9.

Ochlerotatus cataphylla and *O. cantans* are also associated with low water temperatures, which allow them to develop early in spring when water is cold in North European countries, emerging early in May (Becker et al., 2003; Wegner, 2009b), which is consistent with our data as the majority of specimens were found in April. Conversely, higher water temperatures were preferred by species belonging to the *Culex* and *Culiseta* genera. These species develop during summertime with higher water temperatures (except *C. morsitans*). The preference of *Culiseta* for high water temperatures is also indicated by other authors (Becker et al., 2003), who state that, for example, *C. annulata* in Europe is a widespread species with a long season (Becker et al., 2003; Wegner, 2009b). Though in our study, they were found in June–August at low density. *Culiseta morsitans* larvae can develop in various water bodies; they overwinter in the larval stage and pupate only after spring (Becker et al., 2003). However, Oliver and Howard (2011) reveal that northern *C.*

morsitans overwinter in the egg stage, and larvae of this species need high water temperatures to develop. In our research, they were present in April (more than a third of all found) and then reappeared from June through September.

Culex territans and *O. punctor* both showed statistically significant relationships with sampling time, but these relationships were the opposite. While *O. punctor* was found only in April, with an average water temperature of 8.2 °C, *Culex territans* was found in July–August, with an average water temperature of 15.8 °C. Becker et al. (2003) noted that *O. punctor* is a snow-melt mosquito species that starts development early in spring, while *C. territans* may occur from early spring until September, with its peak being in late summer. Some mosquito species (*C. territans*, *Culiseta annulata*, *O. flavescens*, and *O. sticticus*) were found only in water bodies with no nitrates and nitrites detected. The same was observed for *Culex pipiens* as Dowling et al. (2013) revealed that *C. pipiens* preferred to oviposit in containers with lower nitrate content. *Ochlerotatus cantans* were found more abundantly in water bodies with a higher amount of nitrates, as shown by both the multiple regression analysis and the RDA.

According to Becker et al. (2003) and Wegner (2009b), some mosquito species are univoltine (monocyclic), such as the early spring species *O. cataphylla*. This is in agreement with our results, as the majority of *O. cataphylla* larvae were found in April, with some found in May. Similarly, *O. annulipes* is known to be an univoltine species, which coincides with our observations, as this species was detected in April–May, but at low density, differing from what was expected since this species has been recorded as the most abundant in Central Europe (Becker et al., 2003). It is known that *O. cantans* has one generation in the northern part of Europe, but there can be two generations in other parts (Becker et al., 2003; Wegner, 2009b). Our results showed two clear generations of *O. cantans*: the first one in April–May and the second one in August–September (Table 3.1.1.4.). In northern parts of Europe, *Culex territans* has only one generation per season (Wegner 2009b), but in Lithuania, it seems to have quite a long season with multiple generations, as larvae of this species were found from June to August. According to Wegner (2009), *O. sticticus* is considered univoltine, while Becker et al. (2003) stated that it is multivoltine. We found this species in April–May and in July, while no mosquito larvae were found in June, suggesting that at least two generations developed during the season. Wegner (2009) noted that *O. punctor* was a multivoltine species, but in our study, it was found exclusively in April.

Aedes cinereus is a widespread species in the Holarctic region that can have several generations in Lithuania (Table 3.1.1.4.). In the northern part of Europe, it has one generation, but further south, it can have multiple generations a year (Wegner, 2009b). They hatch a little later because their larval development requires a higher temperature than typical snow-melt species (Becker et al., 2003). This might explain why they were found in April but peaked only in May, aligning with our larval stage findings, where the average temperature for *A. cinereus* larvae were higher (12.0 °C) than that of *O. cantans* (8.7 °C) and *O. punctor* (8.2 °C). *Aedes vexans* is a multivoltine species considered to be a "summer species" because its optimal development temperature is known to be around 30 °C (Becker et al., 2003; Wegner, 2009b). This coincides with our results, as these mosquitoes were found in small numbers in April–May and reached their peak in July when the average temperature was the highest (Table 3.1.1.4.).

Mosquito species found together in the same samples (Table 3.1.1.2.) have also been mentioned in other studies (Khalin and Aibulatov, 2020). *Aedes cinereus* can be found together with *O. cantans*, *A. vexans*, *O. behningi*, and *O. cataphylla*; *O. cantans* with *O. communis*, *O. diantaeus*, and *O. punctor*; *O. cataphylla* was found together with *O. annulipes* and *O. communis*; *Culex torrentium* can be found together with *Anopheles claviger* and *Culiseta alaskaensis* (Ludlow); *O. intrudens* was found together with *O. punctor*.

Two anopheline species were found during our study (Table 3.1.1.1.). *Anopheles claviger* can transmit human malaria (Okech et al., 2007; Sallum, 2000). Although anophelines were not very numerous in this study, monitoring their distribution and density is crucial because they tend to fly into buildings, spend their daytime in shady areas, and feed during the night. They are active from May until October, sometimes until November, depending on the season (Wegner, 2009b). Since 1956, no local malaria transmission has been recorded in Lithuania (Žygutienė et al., 1999), but imported cases are being registered (data from Lithuania's National Public Health Centre Under the Ministry of Health).

Culex pipiens is considered to be one of the most common mosquito species distributed throughout the Holarctic region and a main vector of West Nile virus (Wegner, 2009b; Weitzel et al., 2011). However, in our study, they were rare, comprising only 2.3% of all collected and identified mosquito larvae. More widespread and detailed research is needed to determine if this species is not as common in Lithuania as in other European countries. It cannot be ruled out that the most favorable larval habitats for *C. pipiens* were not

found during this study since other studies indicate that adult *C. pipiens* are quite common in Lithuania (Valavičiūtė-Pocienė et al., 2024).

Since most studies focus on mosquito females due to their feeding behavior and vectorial capacity for various pathogens, it is also important to study larval stages. This helps us better understand mosquito diversity and can provide new insights for mosquito control strategies (Gunathilaka et al., 2019; Vantaux et al., 2016; Westbrook et al., 2010).

3.2. Mosquitoes naturally infected with avian haemosporidian parasites (Haemosporida)

3.2.1. Results

During 2021–2023, a total of 2820 mosquitoes were processed to search for haemosporidian parasites. In 2021, 1145 mosquitoes were pooled into 418 pools for molecular testing. In 2022, 837 individual mosquitoes were dissected and processed, and in 2023, 838 individual mosquitoes were processed. Overall, 14 mosquito species from the genera *Aedes*, *Anopheles*, *Coquillettidia*, *Culex, Culiseta*, and *Ochlerotatus* were identified and tested (Figure 3.2.1.1.). In 2021, all mosquitoes were randomly selected for investigation regardless of species. In 2022 CDC traps baited with CO_2 were used and less mosquitoes belonging to genus *Ochlerotatus* were collected. After these two years results, since haemosporidian parasite prevalence in *Ochlerotatus* spp. mosquitoes was very low, it was decided to eliminate mosquitoes of this genus from further research. Amongst investigated species the most numerous was *Coquillettidia richiardii* (25.2%), followed by *Ochlerotatus sticticus* (22.2%), *Culex pipiens* (19.6%), and *Aedes cinereus* (18.6%). The least common species were *Culex territans* (0.04%), *Culiseta alaskaensis* (0.07%), *Aedes vexans* (0.1%), and *O. communis* (0.1%).

During this research, five mosquito species were found to be infected with haemosporidian parasites (Table 3.2.1.1.). Eight haemosporidian species were detected: five *Plasmodium* species (*Plasmodium ashfordi* Valkiūnas et al., 2007 pGRW02, *Plasmodium homonucleophilum* Ilgūnas et al., 2013 pSW2, *P. circumflexum* pTURDUS1, *Plasmodium matutinum* Huff, 1937 pLINN1, and *P. vaughani* pSYAT05) and three *Haemoproteus* species, which illustrated abortive development in non-competent vectors, as mosquitoes are not vectors of *Haemoproteus* parasites (Table 3.2.1.1.).

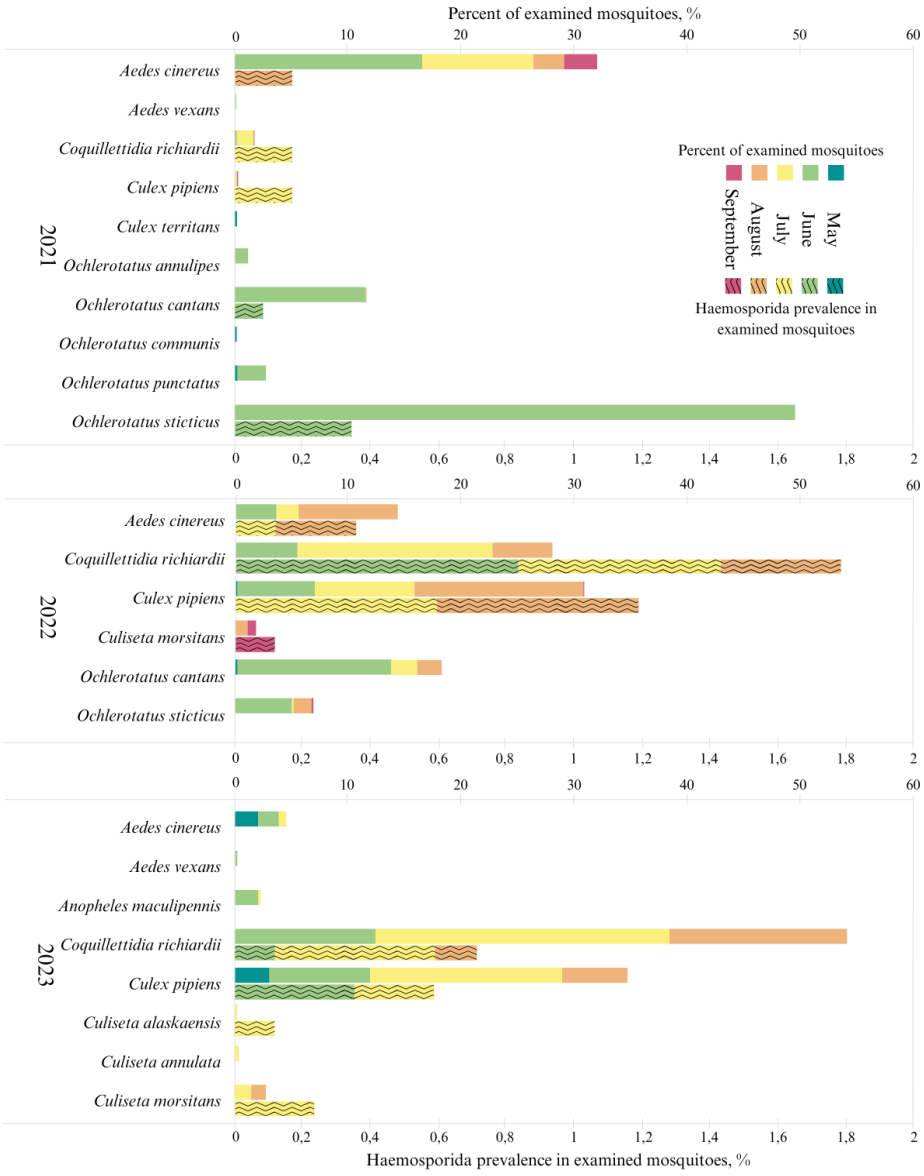

Figure 3.2.1.1. Percentage of examined mosquitoes and haemosporidian parasite prevalence in certain mosquito species in May-September, 2021-2023.

Table 3.2.1.1. Parasite species and genetic lineages detected in different mosquito species and their sampling date.

Detected parasite species	**Genetic lineages of detected parasites**	**Mosquito species where the parasite was found**	**Date of mosquito trapping (number of PCR-positive mosquitoes)**
P. ashfordi	pGRW02	*Ochlerotatus sticticus*	06.08.2021. (1)
P. homonucleophilum	pSW2	*Aedes cinereus*	08.03.2021. (1)
			08.16.2021. (1)
		Coquillettidia richiardii	07.12.2021. (1)
			07.20.2021. (1)
		Culex pipiens	07.12.2021. (2)
		O. sticticus	06.08.2021. (1)
P. circumflexum	pTURDUS1	*O. sticticus*	06.03.2021. (1)
			06.08.2021. (1)
P. matutinum	pLINN1	*A. cinereus*	08.17.2022. (1)
			08.17.2022. (1)
		C. pipiens	08.30.2022. (1) 06.21.2023. (1)
			07.14.2023. (1)
P. vaughani	pSYAT05	*C. richiardii*	08.16.2023. (1)
		C. pipiens	08.30.2022. (1)
Haemoproteus majoris	hWW2	*O. cantans*	06.08.2021. (1)
H. brachiatus	hLK03	*C. pipiens*	06.21.2023. (1)
Haemoproteus asymmetricus Valkiūnas et al., 2021	hTUPHI01	*C. pipiens*	06.21.2023. (1)

If samples tested positive for haemosporidians using PCR, then the salivary gland preparations of those mosquitoes were analyzed. In one preparation of salivary glands of *Culex pipiens* (collected on August 30, 2022) sporozoites of *Plasmodium matutinum* pLINN1 (Figure 3.2.1.2.) were detected. Sporozoites during microscopical analysis were distinguished by their elongated, tapering shape at the ends. This finding adds information to the possible vectorial capacity of *C. pipiens*, indicating that this mosquito species can support the sporogonic development of *P. matutinum* pLINN1. The sporozoites of *P. matutinum* pLINN1 varied in length from 8.1 to 11.2 µm (mean 9.6 µm, SD ±0.7), in width from 0.6 to 1.0 µm (mean 0.8 µm ±1.0), and in area from 4.3 to 9.5 µm² (mean 6.9 µm² ±1.2).

The prevalence of haemosporidian parasites in wild-caught mosquitoes was 2.0% (55 samples were positive by PCR). The prevalence varied across different years, from 0.6% in 2021 to 3.5% in 2022. It also fluctuated between

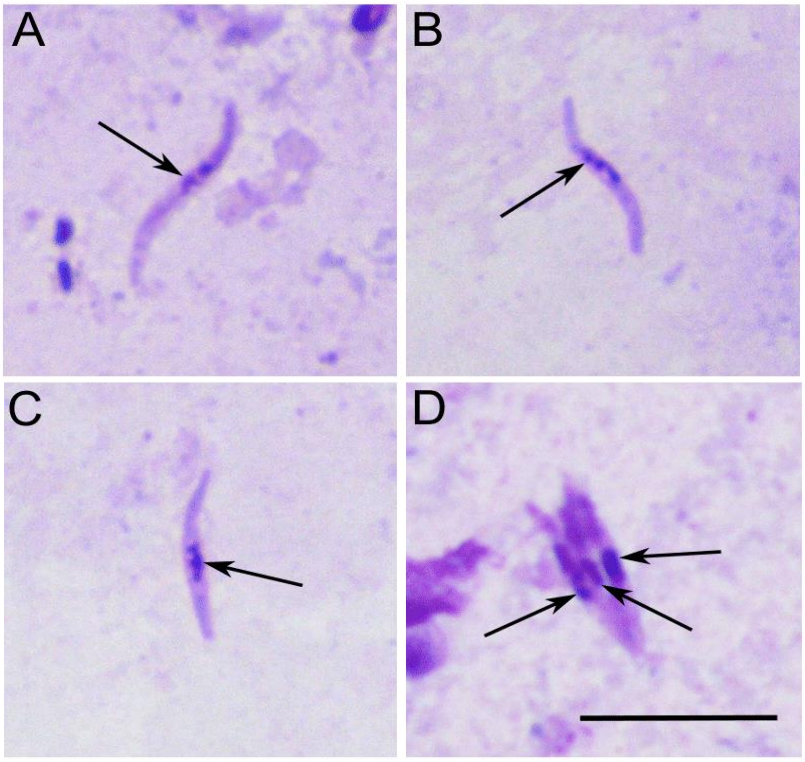

Figure 3.2.1.2. Sporozoites of *Plasmodium matutinum* cytochrome *b* lineage pLINN1 in salivary gland preparation of *Culex pipiens*. Parasite nuclei are indicated by arrows. Scale bar: 10 µm. (Original, photo C. R. F. Chagas).

sampling months, with July (3.1%) and August (2.6%) being the peak months (Chi-square, χ^2 = 11.85, *df* = 4, *P* < 0.02). Different mosquito species also showed varying prevalence rates (Chi-square, χ^2 = 56.32, *df* = 13, *P* < 0.00), with *Coquillettidia richiardii* and *Culex pipiens* being the most infected species at 3.5% (25 PCR-positive mosquitoes) and 2.9% (16 PCR-positive mosquitoes), respectively (Figure 3.2.1.1.). Species from another genus – *Culiseta*, also showed a relatively high infection rate (MIR – 8.7%), although only 46 female mosquitoes were caught and tested. No infected mosquitoes were found in the species *Aedes vexans*, *Anopheles maculipennis*, *Culex territans*, and *Ochlerotatus communis*, possibly due to the low number of mosquitoes tested: three, twenty, one, and three, respectively.

In this study, 35 haemosporidian parasite sequences obtained from mosquitoes in their chromatograms exhibited double peaks, indicating possible infections with more than one haemosporidian parasite. Multiplex PCR confirmed co-infections in 16 out of these 35 cases: eight *Coquillettidia richiardii* specimens were infected with *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* simultaneously; four *C. richiardii* specimens were infected with *Plasmodium* and *Haemoproteus*; and three *C. richiardii* and one *Culex pipiens* specimens had DNA of *Plasmodium* and *Leucocytozoon*. Contig sequences could not be obtained from 19 samples, despite multiple attempts and positive PCR results for haemosporidian DNA (Figure 3.2.1.3.).

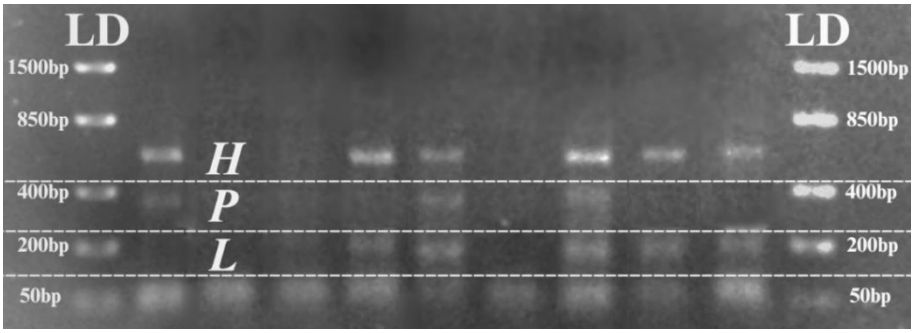

Figure 3.2.1.3. Agarose gel electrophoresis of multiplex PCR products. *H* – *Haemoproteus* (533 bp); *P* – *Plasmodium* (378 bp); *L* – *Leucocytozoon* (218 bp); LD - ladder. (Original, photo K. Valavičiūtė-Pocienė).

3.2.2. Discussion

The detection of *Plasmodium matutinum* (pLINN1) sporozoites in the salivary glands of wild-caught *Culex pipiens* mosquitoes is the most significant finding of this study. This confirmation of the sporozoite stage via microscopy, combined with molecular methods, demonstrates the possible

vectorial capacity of *C. pipiens* for this parasite lineage. Such an integrative approach, combining microscopy and molecular techniques, remains relatively rare in mosquito research but is crucial for confirming the complete development of parasites within vectors (Bernotienė et al., 2019; Chagas et al., 2022, 2024; Kim and Tsuda, 2015; Žiegytė et al., 2021, 2022, 2023).

Previous studies have detected *P. matutinum* in mosquitoes using PCR methods (Ferraguti et al., 2013a; Kimura et al., 2010; Martínez-de la Puente et al., 2015; Schoener et al., 2017; Zélé et al., 2014), but they lacked confirmation of the sporozoite stage in wild-caught mosquitoes. Experimental infections with *P. matutinum* had suggested the potential for the development in *C. pipiens*, *Culex tarsalis*, and *Culex stigmatosoma* (Garnham, 1966), yet field confirmation for this particular genetic lineage was missing until now (Valkiūnas et al., 2017).

The results suggest that *Aedes cinereus*, *Coquillettidia richiardii*, *Culex pipiens*, *Culiseta alaskaensis*, *Culiseta morsitans*, *Ochlerotatus cantans*, and *O. sticticus* can be considered as potential vectors of haemosporidian parasites in Lithuania and probably other temperate zones. The study also reported avian *Plasmodium* and *Haemoproteus* species in these mosquitoes, confirming that these mosquitoes had blood meals on birds and providing knowledge in the mosquito feeding preference on certain bird species. All detected haemosporidian lineages are new records in insects in Lithuania (Bernotienė and Valkiūnas, 2016).

Plasmodium homonucleophilum (pSW2) was the most common parasite lineage detected; it was previously reported in *C. pipiens* in Switzerland (Glaizot et al., 2012) and in various bird species across Europe and Asia (Nourani et al., 2020). *Plasmodium matutinum* (pLINN1) DNA was found in five mosquitoes in this research, and this parasite is widely distributed in Europe, the USA, New Zealand, and Japan in bird species from 16 families (http://130.235.244.92/MalAvi/; accessed April 24, 2024). *Plasmodium circumflexum* (pTURDUS1) was detected in *O. sticticus* and previously in *C. pipiens* in Switzerland (Glaizot et al., 2012). *Plasmodium ashfordii* (pGRW02) was detected in *O. sticticus* for the first time; previously it was found in birds across Europe, Africa, and Asia. *Plasmodium vaughani* (pSYAT05) was found in *Coquillettidia richiardii* and *Culex pipiens*, with a widespread presence in various mosquito species and birds globally (http://130.235.244.92/MalAvi/; accessed April 24, 2024).

The detection of *Haemoproteus* spp. parasites in *C. pipiens* and *O. sticticus* mosquitoes shows that these mosquito species fed on infected birds. However, there is no evidence that mosquitoes can support the sporogonic

development of *Haemoproteus* parasites, likely indicating abortive development and suggesting possible natural model host-parasite associations for future investigation of mechanisms of abortive development of haemosporidians in vectors. Worth mentioning is the fact that during experimental studies of *Haemoproteus* spp. abortive development in *Ochlerotatus cantans* mosquitoes, infected individuals had a greater mortality rate then the control group (Valkiūnas et al., 2013).

The prevalence of haemosporidian parasites in wild-caught mosquitoes varies across different studies. In Madagascar – 5.02% (Schmid et al., 2017), while in Austria 6.43% of pools were positive (Schoener et al., 2017). Studies in Turkey showed a 10.7% prevalence in tested pools (Inci et al., 2012). In this study, the overall prevalence was 2.0%, with a high rate of co-infections (30.7%) among PCR-positive samples. *Culex pipiens* and *Coquillettidia richiardii* had the highest prevalence of haemosporidian parasite DNA.

Culex pipiens is an important vector for avian malaria parasites due to its ornithophilic nature (Farajollahi et al., 2011; Martínez-de la Puente et al., 2015). The presence of *Plasmodium* spp. DNA in *Culex* mosquitoes is well-documented (Kim and Tsuda, 2015), but confirmation of sporozoite stages remains rare (http://130.235.244.92/MalAvi/; accessed April 24, 2024; Nourani et al., 2020). *Coquillettidia* mosquitoes also involved in avian malaria transmission, with studies showing high prevalence rates (Njabo et al., 2009, 2011; Schoener et al., 2017).

During this study mosquito females sampling methodology was changed. In 2021 an entomological sweeping net was used, collected mosquito abdomens were pooled for PCR testing. In 2022 and 2023 CDC traps baited with CO_2 were used, while mosquitoes were processed individually. This shift improved the capture success and detection of infected individuals. These changes did not reduce species diversity of caught mosquitoes, but this way numbers of caught target species mosquitoes (singled out by the results of first year) increased. These findings suggest that *Ochlerotatus* mosquitoes might play a minor role in avian haemosporidian transmission, and future research should focus on species of *Aedes*, *Culex*, *Culiseta*, and *Coquillettidia* genera.

This study underscores the importance of integrating microscopy with molecular methods to add information in understanding of the vectorial capacity of mosquitoes. The detection of *P. matutinum* (pLINN1) sporozoites in *C. pipiens* salivary glands confirms this species as a likely vector, highlighting the need for continued surveillance and integrative research approaches in vector studies.

3.3. Trypanosomatida and Haemosporida parasites in *Culex pipiens* mosquitoes and investigation of hibernating *C. pipiens* mosquitoes

3.3.1. Results

Overall, 1037 female *Culex pipiens* mosquitoes were processed: 556 mosquitoes were collected during the warm season (infections with Haemosporidian parasites discussed in section 3.2.1.) and 481 from wintering sites. No mosquitoes were found to be harboring haemosporidians and trypanosomatids in same individual.

Molecular analysis revealed that amongst active mosquitoes, 16 out of *C. pipiens* 556 mosquitoes (2.9%) were infected with haemosporidian parasites. The identified haemosporidian species and genetic lineages included: *Plasmodium homonucleophilum* (pSW2), *P. matutinum* (pLINN1), *P. vaughani* (pSYAT05), *Haemoproteus brachiatus* (hLK03), and *H. asymmetricus* (hTUPHI01) (Table 3.2.1.1.). The highest prevalence of

Table 3.3.1.1. Trypanosomatidae species found in *Culex pipiens* mosquitoes, with collection date and site given. RP – Regional Park; BG – Botanical Garden.

Collection date	Collection site	Microscopy positive	Trypanosomatid species
05.25.2022.	Verkiai RP	-	*Trypanosoma culicavium*
06.29.2022.	VU Kairėnai BG	$+^{s,m}$	*T. culicavium*
06.29.2022.	VU Kairėnai BG	-	*T. culicavium*
07.14.2022.	VU Kairėnai BG	-	*T. theileri*
08.03.2022.	VU Kairėnai BG	$+^{s,m}$	*T. trinaperronei*
08.03.2022.	VU Kairėnai BG	-	*T. theileri*
08.30.2022.	VU Kairėnai BG	$+^{s,m}$	*T. culicavium*
07.18.2023.	VU Kairėnai BG	$+^{m}$	*T. culicavium*
07.18.2023.	VU Kairėnai BG	-	*T. theileri*
12.07.2023.	Verkiai RP[1]	$+^{m}$	Monoxenous trypanosomatid
12.08.2023.	Antakalnis Bunkers	-	Monoxenous trypanosomatid

1 Cellar of water tower.

s Salivary gland's preparation.

m Midgut preparation.

haemosporidian parasites in *C. pipiens* mosquitoes was detected in July (3.6%) and August (2.9%). None of the hibernating mosquitoes were found to be infected with haemosporidian parasites. The difference between prevalence of haemosporidian parasites between active (collected during warm period) and hibernating mosquitoes was significant (Fischer's exact test, $P = 0.00$).

DNA of trypanosomatids was found in nine out of 556 female *C. pipiens* mosquitoes collected during the warm period (1.6%), and two infected mosquitoes were collected from wintering sites (0.4%). We identified trypanosomatids based on the similarity of the obtained sequences to the ones deposited in GenBank and BOLD systems. The difference between prevalences of *Trypanosoma* parasites between active (collected during warm period) and hibernating mosquitoes was significant (Fischer's exact test, $P = 0.00$).

Trypanosoma culicavium Votýpka et al., 2012 (sequence showed 99.14% similarity to the sequence deposited in GenBank – OM509727) was found in five mosquitoes; *Trypanosoma theileri* Laveran 1902 was identified in three mosquitoes; *Trypanosoma trinaperronei* Garcia et al., 2020 (sequence showed 99.86% similarity to the sequence deposited in GenBank – MN752212) was found in one mosquito. Two specimens were infected with monoxenous trypanosomatids (sequences showed 99.87 and 99.51% similarity to the sequence deposited in GenBank – OP748978) (both mosquitoes were collected from wintering sites) (Table 3.3.1.1., Figure 3.3.1.1.). The highest prevalence of trypanosomatid infections was observed in May (3.5%).

Microscopically, *T. culicavium* was detected in salivary gland and midgut preparations of *C. pipiens* (Figure 3.3.1.2.A). *Trypanosoma trinaperronei* was also detected in both salivary gland and midgut preparations of *C. pipiens* (Figure 3.3.1.2.B). Monoxenous trypanosomatid was found only in midgut preparation (Figure 3.3.1.2.C). *Trypanosoma theileri* was not detected in mosquito salivary gland or midgut preparations.

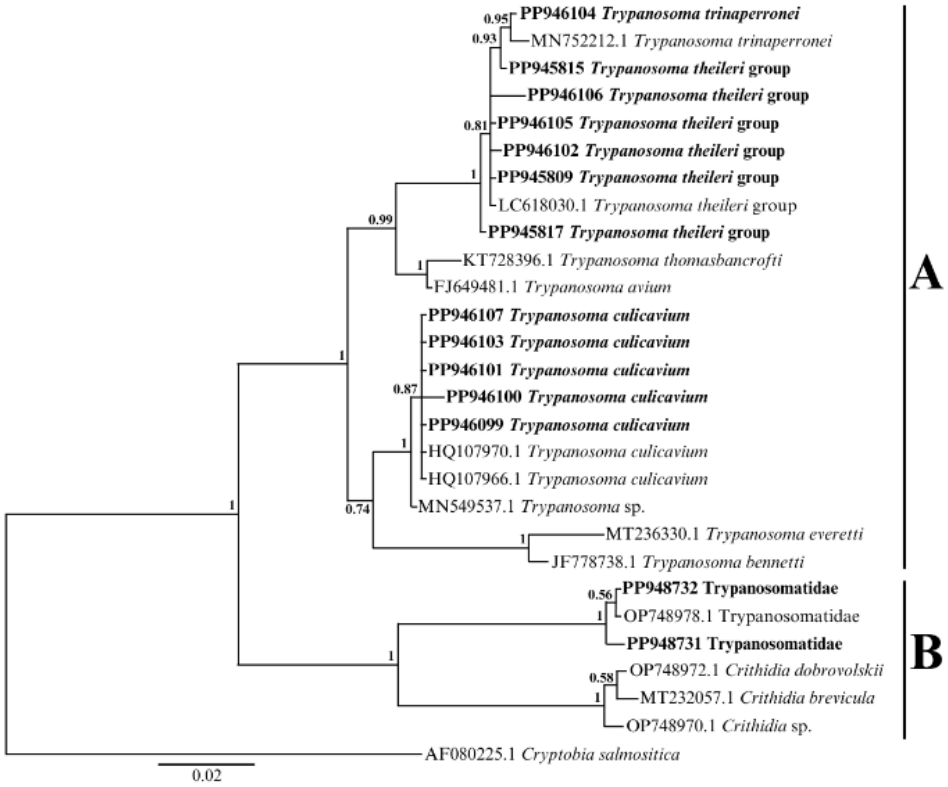

Figure 3.3.1.1. Bayesian phylogenetic tree of *Trypanosoma* (A) and other Trypanosomatidae (B) using fragments of 18S rRNA. The tree was rooted using *Cryptobia salmositica*. Samples obtained during this investigation and their Genbank accession numbers are given in bold text.

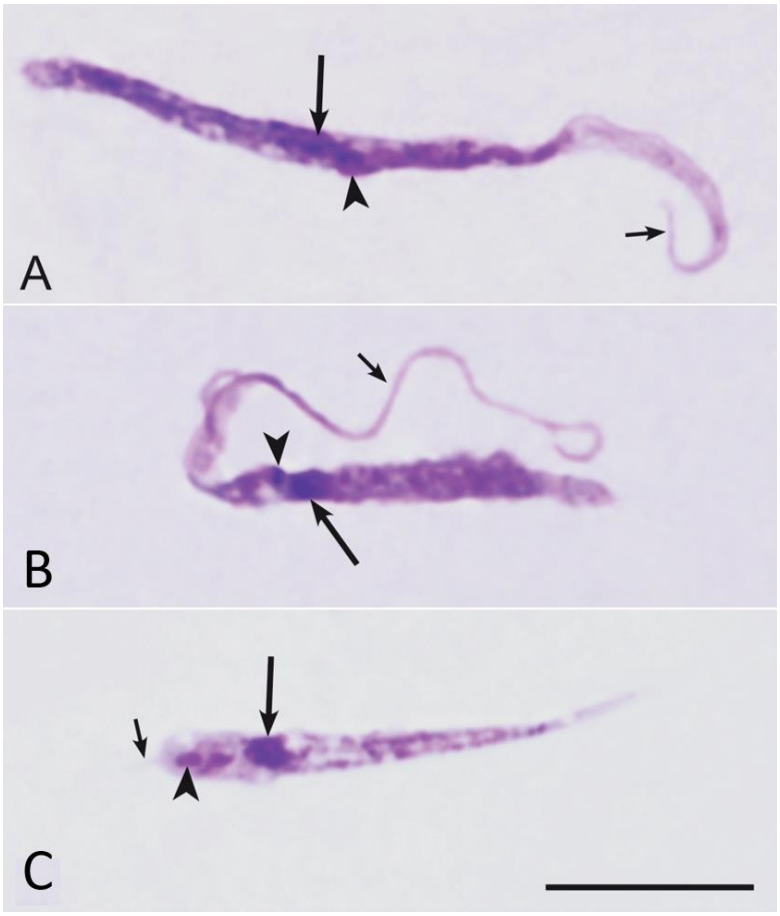

Figure 3.3.1.2. Trypanosomes found in *Culex pipiens* midgut preparations: A – *Trypanosoma culicavium* (sequence GenBank accession PP946100); B – *Trypanosoma trinaperronei* PP946104; C – Trypanosomatidae PP948731. Long arrows – parasite nuclei; arrowheads – kinetoplast; short arrow – free flagellum. Scale bar: 10 μm. (Original, photo T. A. Iezhova).

3.3.2. Discussion

Culex pipiens mosquitoes are considered to be primarily ornithophilic (Farajollahi et al., 2011; Martínez-de la Puente et al., 2015). The presence of avian *Plasmodium* DNA in tested *C. pipiens* females indicates that these mosquitoes had fed on birds, as this is the only way mosquitoes can acquire *Plasmodium* parasites. *Trypanosoma culicavium* is an avian trypanosomatid

(Votýpka et al., 2012), further supporting the ornithophilic tendencies of *C. pipiens*, while *T. theileri* (Brotánková et al., 2022) and *T. trinaperronei* (Garcia et al., 2020) develop in mammals, reflecting the mammophilic or opportunistic behavior of these mosquitoes. Blood-sucking insects like *C. pipiens* can serve as bridge vectors between different animal groups, such as acting as a bridge vector for West Nile virus from birds to humans (Hamer et al., 2014). There have been reported cases of atypical human infections where trypanosomes from other animals infect people, causing serious illness or even death, although such transmissions have not yet been detected with mosquitoes acting as vectors (Truc et al., 2013).

There are at least 16 species, and 50 genetic lineages of *Plasmodium* identified in *C. pipiens* mosquitoes, while sporozoites of only 10 *Plasmodium* species and one unclassified genetic lineage (pCXPIP09) have been found in the salivary glands of this mosquito (Supplementary Table 2). Although mosquitoes are a dead end for parasites of other haemosporidian genera (such as *Haemoproteus*), the collection of such data provides new insights into the feeding preferences of mosquitoes. At least four different species and 17 genetic lineages of *Haemoproteus* were found in *C. pipiens* mosquitoes, and one species and genetic lineage of *Leucocytozoon* were also detected (Supplementary Table 2). The prevalence of haemosporidian parasites in *C. pipiens* mosquitoes varies between 0.04% and 6.6% in different studies (0.04% – (Ventim et al., 2012); 0.52% – (Ejiri et al., 2009); 0.61% – (Synek et al., 2013); 3.08% – (Kim and Tsuda, 2010); 6.6% – (Glaizot et al., 2012)), and our detection of 2.9% prevalence during the warm season falls in the middle of this range.

There is limited data on the detection of haemosporidian parasites in hibernating mosquitoes. *Plasmodium matutinum* (pLINN1) was found in two pools of mosquitoes collected from hibernation sites in November using PCR in Germany (Köchling et al., 2023). This parasite is frequently detected in *C. pipiens* using PCR (Supplementary Table 2), and recently, sporozoites of *P. matutinum* (proven molecularly and microscopically) were detected in the salivary glands of *C. pipiens* mosquitoes (Valavičiūtė-Pocienė et al., 2024), indicating that this parasite successfully completes its sporogonic development in this mosquito species. We concur with the authors (Köchling et al., 2023) that *Plasmodium* parasites may be able to overwinter in hibernating mosquitoes, but additional field studies, combined with experimental observations, are needed to confirm this hypothesis. October–November marks the beginning of hibernation for mosquitoes that overwinter as adult females in the temperate zone of the northern hemisphere (Becker et

al., 2003), and mosquitoes with parasites should survive at least until April. We detected the statistically significant difference in *Plasmodium* parasite prevalence between warm and cold periods in *C. pipiens* mosquitoes.

A high diversity of trypanosomatids has been found in *C. pipiens* mosquitoes worldwide (Supplementary Table 3). Two *Trypanosoma* species are known to be transmitted by these mosquitoes: *T. culicavium*, originally described from the gut of *C. pipiens* (Votýpka et al., 2012; Zídková et al., 2012), and *Trypanosoma thomasbancrofti* Šlapeta et al., 2016 (Fialová et al., 2021; Šlapeta et al., 2016; Zídková et al., 2012), for which *C. pipiens* has been confirmed as a vector (Fialová et al., 2021). Over the years, other trypanosomatids have been periodically detected in this mosquito species, including *T. avium* (Schoener et al., 2019; 2018; Svobodová et al., 2015), *T. theileri* (Brotánková et al., 2022), and various monoxenous trypanosomatids such as several *Crithidia* species: *Crithidia brevicula* Frolov and Malysheva, 1989 (Svobodová et al., 2015), *Crithidia dedva* Kostygov, Malysheva, and Frolov, 2011 (Schoener et al., 2019), *Crithidia fasciculata* Léger, 1902 (Schoener et al., 2018), *Crithidia dobrovolskii* Ganyukova and Frolov, 2020), *Strigomonas* cf. *oncopelti* Lwoff and Lwoff, 1931, *Paratrypanosoma* cf. *confusum* Votýpka and Lukes, 2013, and an unknown lineage of Trypanosomatidae (Kostygov et al., 2022). These findings are noteworthy because the primary vector of *T. avium* is blackflies (Simuliidae) (Votýpka et al., 2002), with *Culicoides* (Ceratopogonidae) as potential vectors (Bernotienė et al., 2020; Kazak et al., 2023) in the wild. It has been reported that among mosquito-transmitted avian trypanosomes, the prevalence of *T. thomasbancrofti* in wild-caught mosquitoes and birds is lower (0.13%) compared to *T. culicavium* (4.5%) (Fialová et al., 2021). The overall prevalence of *T. thomasbancrofti* in wild-caught mosquitoes in Europe is low (Fialová et al., 2021; Schoener et al., 2018; Svobodová et al., 2015); this can explain why we did not detect this parasite species in *Culex* mosquitoes during our study.

Trypanosoma theileri was one of the first mammalian trypanosomatids described, with deer keds (Garcia et al., 2020) and tabanids being the main vectors (Böse et al., 1987). Several lineages of *T. theileri* (TthI and TthII) have been defined based on analyses of Internal Transcribed Spacer (Rodrigues et al., 2006, 2010), and 18S rRNA gene phylogenies (Ganyukova et al., 2018; Garcia et al., 2011; 2020; Jaimes-Dueñez et al., 2018; Pacheco et al., 2018). Although data on the prevalence of this parasite in mosquitoes is scarce (Fialová et al., 2021), recent studies show that the prevalence in *Culex* mosquitoes reaches only 0.05% (while in *Aedes* and *Ochlerotatus* (in the

article refered to as *Aedes*) species it can be as high as almost 22%) (Brotánková et al., 2022). This difference between *T. theileri* prevalences in *C. pipiens* and some *Aedes* and *Ochlerotatus* species might come from feeding preference. *Culex pipiens* more often feed on bird's blood and knowing that *T. theileri* is mammalian trypanosome (Garcia et al., 2020), low prevalence in this mosquito species is not unexpected. In comparison to the 2022 study by Brotánková and colleagues – our findings show a little higher prevalence of *T. theileri* in *Culex* mosquitoes (0.72%), with infections confirmed by PCR but not microscopically. We dissected and prepared midgut samples, but some data suggest that *T. theileri* group trypanosomes may prefer to develop in the hindgut (Brotánková et al., 2022), which could explain the lack of microscopic detection in our study.

Currently, several species of *Megatrypanum* trypanosomes are known to parasitize deer, including *Trypanosoma mazamarum* Mazza, Romana, and Fiora, 1932 (Mazza et al., 1932), *Trypanosoma cervi* Kingston and Morton, 1975 (Kingston and Morton, 1975), *T. stefanskii* (Kingston et al., 1992), and *T. trinaperronei* (Garcia et al., 2020). Along with *T. theileri*, *Trypanosoma melophagium* Flu, 1908, *T. cervi*, and *T. trinaperronei* form a complex known as the *Trypanosoma theileri* complex (Garcia et al., 2011; 2020; Rodrigues et al., 2006). Deer keds (*Lipoptena cervi* (Linnaeus, 1758) and *Lipoptena mazamae* Rondani, 1878) are recognized vectors of *T. trinaperronei* (Garcia et al., 2020), and this parasite had previously never been found in mosquitoes. We identified one *C. pipiens* mosquito infected with *T. trinaperronei* both molecularly and microscopically. The sequence obtained shows 99.7% similarity with a sequence deposited in GenBank (MN752212), with the type host being the white-tailed deer (*Odocoileus virginianus* (Zimmerman, 1780), Ruminantia, Cervidae) (Garcia et al., 2020).

Recently, an unknown lineage, identified as a new previously not recorded monoxenous trypanosomatid, was discovered by Kostygov et al. (2022) in hibernating *Culex torrentium* mosquitoes. Both *Culex torrentium* and *C. pipiens* feed on birds and mammals (including humans), which may make them potential bridge vectors for transmitting zoonotic pathogens from birds to humans (Jansen et al., 2019). In our study, two hibernating *C. pipiens* females were found to be infected with an unknown trypanosomatid that showed 99.87% and 99.51% similarities to the parasite found in *C. torrentium* (Kostygov et al., 2022). Despite the nearly doubling of described monoxenous trypanosomatid genera over the past two decades, there is still limited information on trypanosomatid diversity in various insect groups, including Diptera (Frolov et al., 2021). While monoxenous trypanosomatids are

generally considered non-pathogenic, some evidence suggests they may negatively impact insect fitness, although this has only been investigated in a few insect species (Frolov et al., 2021). If monoxenous trypanosomatids are pathogenic to their insect hosts, they could have effect on insect populations (Kostygov et al., 2021). These insect parasites are not directly related to blood-feeding habits and can infect hosts through various means (e.g., feeding on infected prey or feces, or via contaminated substrates like sugar meals) (Frolov et al., 2021; Votýpka et al., 2021).

There appears to be insufficient data on the seasonality of prevalence of avian trypanosomes in vectors. Vanderplank (1947) described seasonal variations in the number of mammals with *Trypanosoma* infections in their blood, noting that infections were highest during the rainy seasons and the months of March–April and November–December (Vanderplank, 1947). However, Pori et al. found no seasonal variations in avian trypanosome infection prevalence among a total of 685 birds from 87 species (Pori et al., 2023).

It is important to mention that during this research trypanosomes was found in midgut and salivary gland preparations, but the latter might be a contamination from the gut. Further investigation is necessary to confirm trypanosomes in salivary glands.

In summary, our investigation did not detect hibernating *Culex pipiens* mosquitoes infected with avian haemosporidians or avian *Trypanosoma* parasites. During the warm season, the prevalence of these parasites was 2.9% for haemosporidians and 2.5% for avian trypanosomatid parasites. Only monoxenous trypanosomatids were detected in hibernating *C. pipiens* mosquitoes. However, experimental studies are needed to determine whether certain parasite species can survive in mosquitoes through the winter or if infected insects do not survive until spring.

3.4. Biting midges naturally infected with avian blood parasites (Haemosporida)

3.4.1. Results

During years 2021–2022, 2533 parous *Culicoides* females were collected and dissected (580 biting midges in 2021, 1953 biting midges in 2022). Among them 13 species were identified, all of them were previously detected in Lithuania. During this research, the most abundant biting midges were those belonging to *Culicoides obsoletus* group – 26.0% (31.9% (2021) and 23.7%

(2022)), *C. pictipennis* – 22.4% (0.3% (2021), 30.3% (2022)), and *C. kibunensis* – 22.3% (2021 – 19.7%; 2022 – 23.1%). In 2021 other abundant species were *C. festivipennis* (15.3%) and *C. punctatus* (11.9%). In 2021 *C. chiopterus, C. deltus,* and *C. pictipennis* were the least common species while *C. pulicaris* and *Culicoides circumscriptus* were the least common in 2021 and *Culicoides reconditus* was rare during both years (Table 3.4.1.1.). Eight specimens were not identified to the species level, so they were referred to as *Culicoides* sp. (Table 3.4.1.1.).

Table 3.4.1.1. Summary of collected *Culicoides* biting midges, with reported *Haemoproteus* and *Plasmodium* species and lineages in them. The first number indicates the number of PCR positive specimens, the number of positive samples detected both by PCR and microscopy (sporozoites were found) is given in brackets.

	C. chiopterus	*C. circumscriptus*	*C. deltus*	*C. festivipennis*	*C. impunctatus*	*C. kibunensis*	*C. obsoletus* group	*C. pallidicornis*	*C. pictipennis*	*C. pulicaris*	*C. punctatus*	*C. reconditus*	*C. segnis*	*Culicoides* sp.	Total
H. asymmetricus hTUPHI01				1		23 (12)			65 (10)				6 (5)		95 (27)
H. belopolskyi hHIICT1				5 (2)		5 (1)	2	1	1						14 (3)
H. fringillae hCCF3													2 (1)		2 (1)
H. homogeneae hSYAT16									1 (1)						1 (1)
H. homominutus hCUKI1						2 (1)									2 (1)
H. magnus hROFI1												1 (1)			1 (1)
H. majoris hCCF5						2									2
H. majoris hCWT4							1								1
H. majoris hPARUS1							1						1		2
H. majoris hPHSIB1													1 (1)		1 (1)
H. majoris hWW2							1								1
H. minutus hTUCHR01						1									1
H. minutus hTURDUS2						16 (8)			15 (2)				3 (3)		34 (13)

	C. chiopterus	*C. circumscriptus*	*C. deltus*	*C. festivipennis*	*C. impunctatus*	*C. kibunensis*	*C. obsoletus* group	*C. pallidicornis*	*C. pictipennis*	*C. pulicaris*	*C. punctatus*	*C. reconditus*	*C. segnis*	*Culicoides* sp.	Total
H. parabelopolskyi hSYAT01						1 (1)			2 (2)						3 (3)
H. parabelopolskyi hSYAT02						1 (1)			4 (3)						5 (4)
H. syrnii hCULKIB01									1						1
Haemoproteus sp. hCCF2						1									1
Haemoproteus sp. hCIRCUM05													1		1
Haemoproteus sp. hCULKIB02						1									1
Haemoproteus sp. hCULKIB03						1									1
Haemoproteus sp. hCULKIB04						1									1
Haemoproteus sp. hCULKIB05						1									1
Haemoproteus sp. hCULPIC01									1						1
Haemoproteus sp. hCULPIC02									1 (1)						1 (1)
Haemoproteus sp. hHAWF6						1									1
Haemoproteus sp. hSYAT13						1 (1)			1 (1)						2 (2)
Plasmodium sp. pCULFES01					1										1
Plasmodium sp. pCULOBS01							1								1
P. vaughani pSYAT05				2											2
Co-infection				1	1	1	1		4 (1)				1 (1)		9 (2)
Negative	2	1	1	266	159	510	630	68	495	3	114	1	85	8	2343
Total positive	0	0	0	9 (2)	2	59 (25)	7	1	96 (21)	0	0	1 (1)	15 (11)	0	190 (60)
Total sampled	2	1	1	275	161	569	637	69	591	3	114	2	100	8	2533
Prevalence (%)	0	0	0	3.7	0.6	10.4	0.9	1.5	16.2	0	0	16.7	15.6	0	7.5

Regarding study sites, the majority of insects were collected and analyzed at the VU Kairėnai Botanical Garden (42.4%), followed by Puvočiai village

(32.3%), the Verkiai Regional Park (21.2%), and Brinkiškės village (4.1%). The highest number of *Culicoides* species was detected at the VU Kairėnai Botanical Garden (ten species), while the Verkiai Regional Park (seven species) had the lowest number of species (Figure 3.4.1.1.). At the VU Kairėnai Botanical Garden, the most abundant species was *C. kibunensis* (Figure 3.4.1.1.), while in Puvočiai village, biting midges from the *C.*

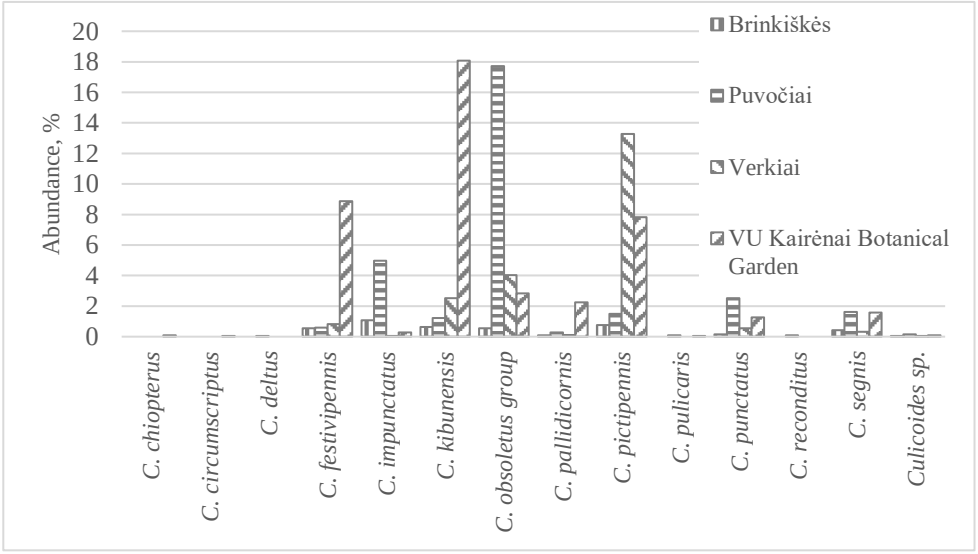

Figure 3.4.1.1. Abundance (%) of *Culicoides* species that were collected and analysed in different sampling sites.

obsoletus complex were more common (Figure 3.4.1.1.), and in the Verkiai Regional Park, *C. pictipennis* was more frequently found (Figure 3.4.1.1.). *Culicoides chiopterus, C. circumscriptus* was collected only at the VU Kairėnai Botanical Garden, and *C. reconditus* was collected only in Puvočiai village. Eight biting midge species were found in low numbers in Brinkiškės village, with *C. impunctatus* being the most abundant (Figure 3.4.1.1.).

Haemosporidian parasite DNA was detected in 190 (36 in 2021 and 154 in 2022) biting midge individuals (5.9% in 2021 and 7.9% in 2022). Four biting midges were positive for *Plasmodium* DNA, 177 specimens were positive for *Haemoproteus* DNA, while the remaining nine specimens had co-infections with parasites of different genera. During this research ten species and 26 genetic lineages belonging to genus *Haemoproteus* were detected and three genetic lineages belonging to genus *Plasmodium*. Out of these lineages six were new (hCULKIB02, hCULKIB03, hCULKIB04, hCULKIB05, hCULPIC01, hCULPIC02) belonging to genus *Haemoproteus* and two new lineages (pCULFES01, pCULOBS01) belonging to genus *Plasmodium* (Table

3.4.1.1.). Notably, 13 of the recovered parasite lineages are still not identified to the species level (Table 3.4.1.1.). Highest infection prevalences were detected in *Culicoides pictipennis* (16.2%), *C. segnis* (15.6%), and *C. kibunensis* (10.4%) biting midge species (Chi-square, $\chi^2 = 153.01$, $df = 13$, $P < 0.00$).

Microscopy analysis of salivary gland preparations from the PCR-positive samples showed the presence of sporozoites in 60 samples (Table 3.4.1.1.); among them, 25 samples of infected *C. kibunensis* biting midges had sporozoites in their salivary gland preparations, 21 samples of *C. pictipennis*, 11 samples of *C. segnis*, two of *C. festivipennis*, and one samples of *C. reconditus* (Table 3.4.1.1.). For the first time, *C. segnis* has been confirmed as a competent host to complete sporogony and thus likely vector for *H. fringillae* hCCF3, *H. majoris* hPHSIB1, *H. asymmetricus* hTUPHI01 (Figure

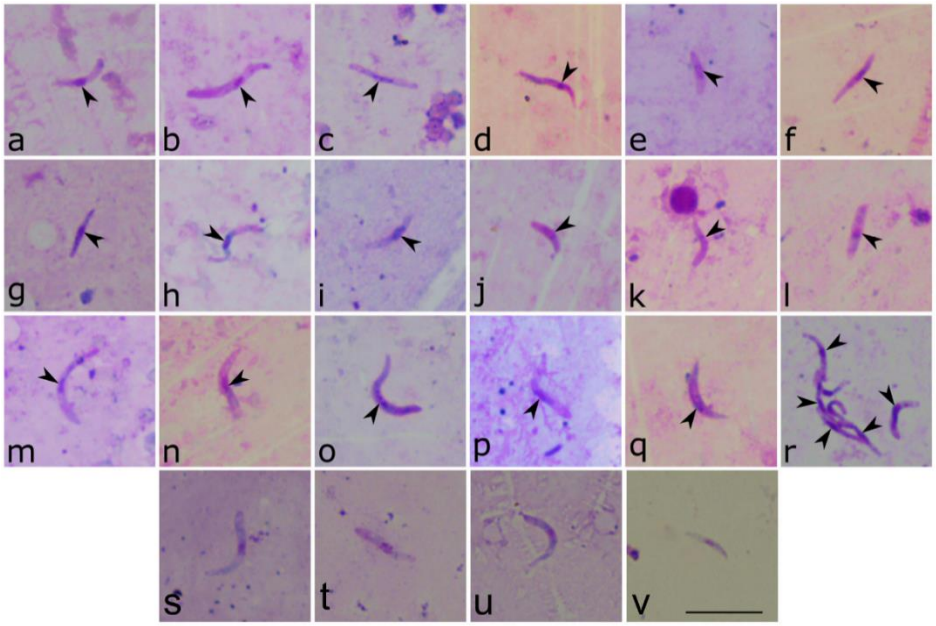

Figure 3.4.1.2. Sporozoites found in the salivary gland preparations of *Culicoides festivipennis* (a), *C. kibunensis* (b,f,h,j,l,o,s,t,u,v), *C. pictipennis* (c,d,g,i,k,m,p,r), *C. reconditus* (e), and *C. segnis* (n,q). These sporozoites belong to *Haemoproteus belopolskyi* genetic lineage hHIICT1 (a), *H. homominutus* hCUKI1 (b), *Haemoproteus* sp. hCULPIC02 (c), *H. homogeneae* hSYAT16 (d), *H. magnus* hROFI1 (e), *H. parabelopolskyi* hSYAT01 (f,g,r) and hSYAT02 (h,i), *Haemoproteus* sp. hSYAT13 (j,k), *H. asymmetricus* hTUPHI01 (l–n,s,u), *H. fringillae h*CCF3 (t) and *H. minutus* hTURDUS2 (o–q,v). Arrowhead indicates sporozoite nucleus. Methanol fixed and Giemsa-stained. Scale bar: 10 μm. (Original, photo C. R. F. Chagas).

3.4.1.2.n), and *H. minutus* hTURDUS2 (Figure 3.4.1.2.q). This is also the first report of *C. kibunensis* being a competent host for completing sporogony and thus likely vector for *H. belopolskyi* hHIICT1, *Haemoproteus homominutus* Valkiūnas et al., 2019 hCUKI1 (Figure 3.4.1.2.b), *H. parabelopolskyi* hSYAT01 (Figure 3.4.1.2.f) and hSYAT02 (Figure 3.4.1.2.h), and *Haemoproteus* sp. hSYAT13 (Figure 3.4.1.2.j). For the first time, *C. reconditus* has been confirmed as a competent host for sporogony and thus likely natural vector of *Haemoproteus* parasites, specifically *H. magnus* hROFI1 (Figure 3.4.1.2.e). *Culicoides pictipennis* was also confirmed as a likely natural vector of *H. parabelopolskyi* hSYAT01 (Figure 3.4.1.2.g), *Haemoproteus* sp. hSYAT13 (Figure 3.4.1.2.k), *Haemoproteus homogeneae* Valkiūnas et al., 2019 hSYAT16 (Figure 3.4.1.2.d), *H. asymmetricus* hTUPHI01 (Figure 3.4.1.2.m), and *H. minutus* hTURDUS2 (Figure 3.4.1.2.p), while *C. festivipennis* had sporozoites of *H. belopolskyi* hHIICT1 (Figure 3.4.1.2.a). Sporozoites of *H. homominutus* hCUKI1, *H. magnus* hROFI1, *H. homogeneae* hSYAT16, *H. parabelopolskyi* hSYAT01, and *Haemoproteus* sp. hCULPIC02 (Figure 3.4.1.2.c) and hSYAT13 (Figure 3.4.1.2.j, k) are being reported for the first time.

In one of the samples, high intensity of sporozoites was detected, with sometimes all in the same field of the microscope (Figure 3.4.1.2.r). One sample presenting a co-infection during PCR was also positive by microscopy (Table 3.4.1.1.). All insects that were PCR-positive for *Plasmodium* DNA were negative by microscopy. It reaffirms that haemosporidians of genus *Plasmodium* is not transmitted by biting midges.

The sporozoites from different *Haemoproteus* parasites and lineages are morphologically very similar (Figure 3.4.1.2.). They are readily recognized in the salivary gland preparations by their elongated shape, with an average length usually exceeding 10 μm, with both ends approximately equally pointed, and a more or less centrally located nucleus. Despite these similarities, some slight morphological differences were noted: sporozoites of *H. homominutus* hCUKI1 (Figure 3.4.1.2.b), *H. asymmetricus* hTUPHI01 (Figure 3.4.1.2.l – n), and *H. minutus* hTURDUS2 (Figure 3.4.1.2.o – q) seem to have a greater length and width compared to the others. On the other hand, sporozoites of *H. parabelopolskyi* hSYAT01 and hSYAT02 (Figure 3.4.1.2.f – i) seem to be thinner and shorter. Due to the differences in the number of sporozoites in positive salivary gland preparations (usually single sporozoites present), their morphometry could not be compared statistically, and it could not be confirmed if these morphological features have any taxonomical importance. For the same reason, a comparison between sporozoites from the

same *Haemoproteus* lineage found in different *Culicoides* species could not be carried out; further studies are needed.

The interaction network between *Culicoides* and *Haemoproteus* lineages (Figures 3.4.1.3.; 3.4.1.4.) showed that, in the wild, *C. kibunensis* is frequently

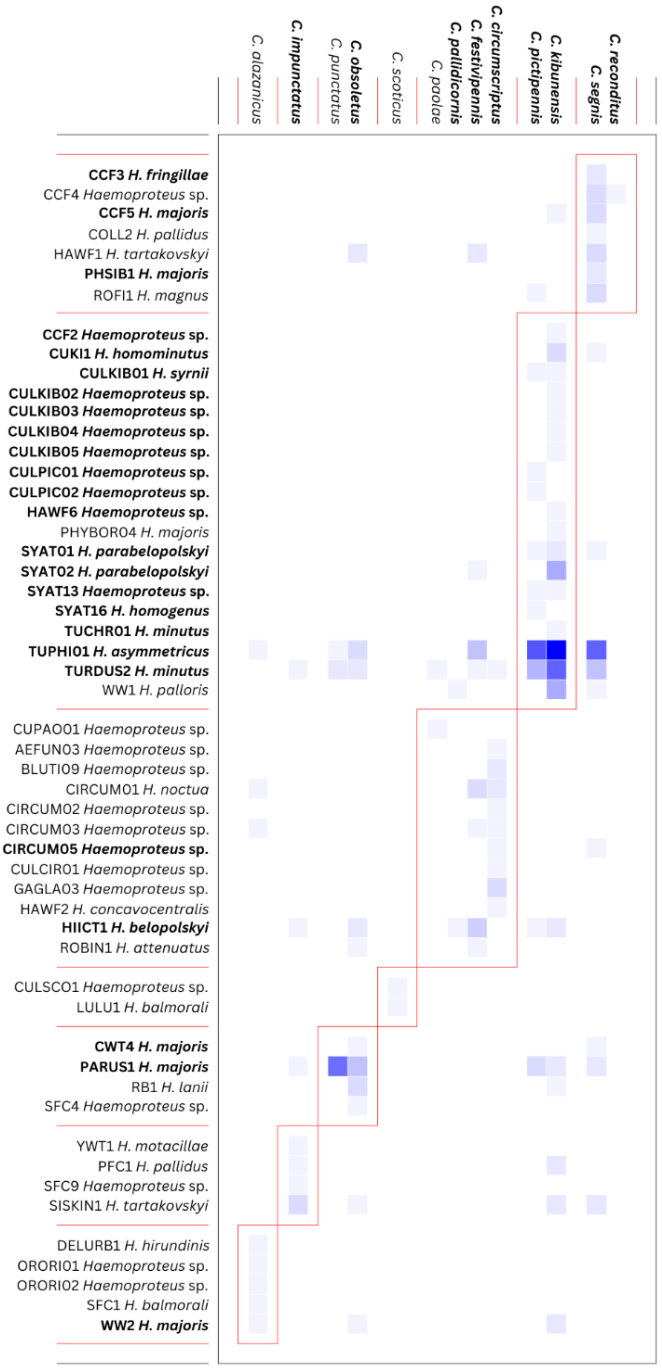

Figure 3.4.1.3. Modularity interaction network between *Culicoides* species (rows) and *Haemoproteus* lineages (columns). The blue squares represent the interactions between insects and parasites; the darker square shows more often registered interactions. *Culicoides* species and *Haemoproteus* genetic lineages determined in this study are marked in bold. Red lines separate different modules.

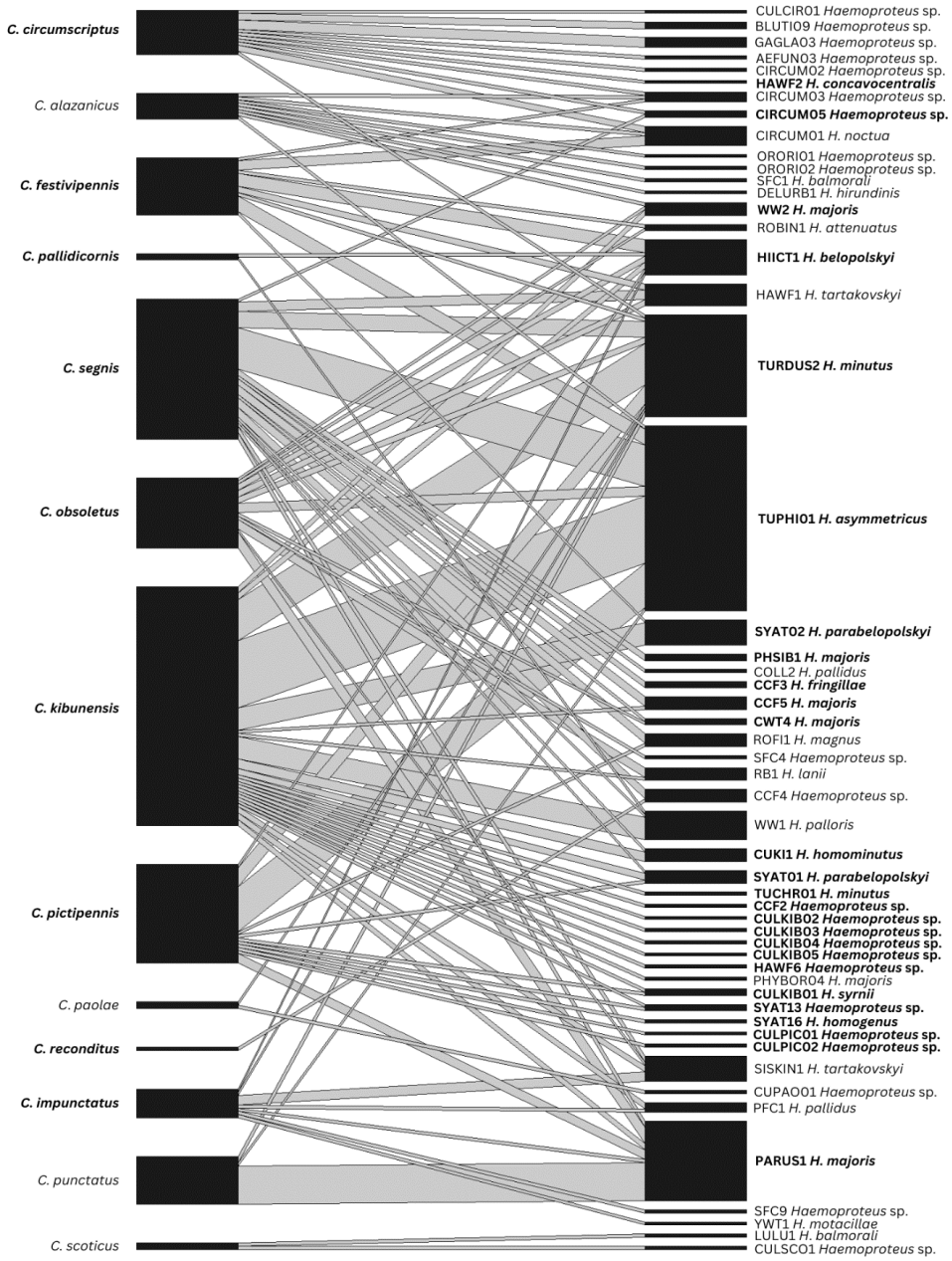

Figure 3.4.1.4. Bipartite interaction network between *Haemoproteus cytb* lineages and *Culicoides* species. The width of the boxes is proportional to the frequency of the occurrence of a particular taxon. The width of connecting lines is proportional to the number of interactions observed between each *Culicoides* species and *Haemoproteus* lineage. *Culicoides* species and *Haemoproteus* lineages investigated and found in this study are marked in bold.

associated with *H. asymmetricus* hTUPHI01 and *H. minutus* hTURDUS2.

Additionally, *H. asymmetricus* hTUPHI01 also presented a high number of interactions with *C. segnis* and *C. pictipennis*. *Culicoides punctatus* mostly interacted with *H. majoris* hPARUS1. *Haemoproteus minutus* hTURDUS2 was found interacting with nine different species of biting midges (Figure 3.4.1.4.), suggesting low specificity in terms of vectors. Most *Culicoides* species presented interactions with several genetic lineages, suggesting that these insects are probable generalists in terms of parasite lineage transmission. *Culicoides kibunensis* is a species with highest number of interactions with *Haemoproteus* lineages having 23 interactions and *C. segnis* having 16 interactions, and *C. obsoletus* group and *C. pictipennis*, each with 11 interactions (Figures 3.4.1.3.; 3.4.1.4.).

Modularity analysis detected seven different modules (Figure 3.4.1.3.), indicating that the transmission of *Haemoproteus* parasites in Europe presents a compartmentalized pattern, with no clear nestedness observed. In other words, different species or groups of species of *Culicoides* are more likely to transmit many groups of *Haemoproteus* lineages.

3.4.2. Discussion

The key results of this study enhance our understanding of the biting midges associations with *Haemoproteus* parasites; that is important for the better understanding of mechanisms of transmission. These findings include the first-time detection of sporozoites of four *Haemoproteus* species (*H. minutus*, H. *asymmetricus*, *H. majoris* and *H. fringillae)* in salivary gland preparations from *C. segnis*, as well as sporozoites of four *Haemoproteus* species (*H. belopolskyi*, *H. homominutus*, *H. parabelopolskyi* and *Haemoproteus* sp.) in *C. kibunensis*, confirming them as likely competent vectors. Our study confirmed *C. reconditus* as a likely natural vector of *H. magnus* parasites for the first time. *Culicoides pictipennis* was confirmed as a competent host for completing sporogony of *H. parabelopolskyi*, *Haemoproteus* sp., *H. homogeneae*, *H. asymmetricus*, *H. minutus* for the first time as well as *C. festivipennis* for *H. belopolskyi*.

Culicoides kibunensis, C. pictipennis, and *C. segnis* are particularly important as possible vectors of *Haemoproteus* parasites, interacting with many *Haemoproteus* species and lineages (Figures 3.4.1.3.; 3.4.1.4.). Conversely, some *Haemoproteus* parasites, such as *H. asymmetricus* hTUPHI01, appear to be restricted to specific biting midge species like *C. segnis*, *C. pictipennis*, and *C. kibunensis*. Additionally, the high number of interactions between *C. kibunensis* and *Haemoproteus* lineages commonly

found in Turdidae birds (hTURDUS2 and hTUPHI01) may indicate a feeding preference of *C. kibunensis* for Turdidae birds.

We identified 13 different species of biting midges in our study (Table 3.4.1.1.). The most abundant species were from the *C. obsoletus* group, *C. pictipennis,* and *C. kibunensis,* consistent with previous reports in this country (Bernotienė et al., 2019; Žiegytė et al., 2021, 2022). Other species, such as *C. fagineus, Culicoides albicans, C. newsteadi, Culicoides fascipennis* (Staeger 1839), *C. circumscriptus, C. pulicaris,* and *C. reconditus,* appear to be rarer (Bernotienė et al., 2019, 2021; Žiegytė et al., 2021, 2022) but we found the latter three species in low numbers (Table 3.4.1.1.). *Culicoides pallidicornis* and *C. punctatus* were notably more abundant in the VU Kairėnai Botanical Garden and Puvočiai village, respectively, but scarce or absent in other localities (Figure 3.4.1.1.). For instance, *C. impunctatus* was more commonly observed in May and June in the Curonian Spit, Lithuania (Žiegytė et al., 2023), but it did not occur during those months and/or at that location in our samples. Notably, we also reported *C. deltus*, a rare species not previously recorded in these studies; although *C. deltus* is found throughout Europe (Mathieu et al., 2012), it seems to be rare in Eastern Europe (Larska et al., 2017). Additionally, *C. impunctatus* is notably abundant in some areas of Lithuania (Valavičiūtė et al., 2020). The prevalence of *C. impunctatus* also varied, with high abundance in some regions, such as Puvočiai and Brinkiškės villages (Figure 3.4.1.1.), aligning with previous findings (Valkiūnas et al., 2002) but differing across regions (Chagas et al., 2022). This variation highlights that species diversity can markedly fluctuate annually and between study sites, underscoring the need for further research across different regions to better understand insect diversity and their role as *Haemoproteus* parasite vectors.

The overall prevalence of *Haemoproteus* lineages in *Culicoides* biting midges was 7.5%, similar to findings in other studies conducted in Lithuania (Bernotienė et al., 2019 – 7%; Žiegytė et al., 2022 – 5.8%). However, this prevalence is higher than in Bulgaria (approximately 2%) (Bobeva et al., 2013, 2014) and Curonian Spit (1.7%) (Bernotienė and Valkiūnas, 2016), but lower than in Spain (13.4%) (Ferraguti et al., 2013b). These differences may reflect variations in study sites, insect density, species diversity, feeding preferences, bird species diversity, parasite prevalence in bird populations, and study timing.

Regarding the *C. obsoletus* group - the most abundant biting midge in this study - we found a low prevalence of infections (only 0.9%). A previous study in one of our sampling areas (Verkiai Regional Park) did not report

infections in this species (Žiegytė et al., 2021). However, another study in the same area in 2016 reported a prevalence of approximately 6% (Bernotienė et al, 2019), similar to that found in the Curonian Spit (Žiegytė et al., 2022). This highlights how different infection prevalences can be, varying by study site and time of year, complicating the understanding of host-parasite-vector relationships. Although *Haemoproteus* DNA is frequently found in *C. obsoletus*, indicating it feeds on birds despite its preferable mammophilic behavior (Bartsch et al., 2009; Pettersson et al., 2013), *Haemoproteus* sporozoites have not previously been reported in this species as well as in present study.

Notably, while 2021 sampling indicated *C. pictipennis* as one of the least abundant species (Chagas et al., 2022), in 2022 it was found to be the most abundant and present in all study sites (Table 3.4.1.1., Figure 3.4.1.1.) and overall abundance was 23.4%. This discrepancy could be due to differences in trapping dates: the 2021 biting midge collection began at the end of June, while 2022 collection started at the beginning of June, when these insects are more prevalent. Fluctuations in insect populations are also possible, especially in temperate zones where density changes are more pronounced and influenced by climatic conditions (Bernotienė et al., 2021; Cuéllar et al., 2018; Mullen and Murphree, 2019; Saroya et al., 2021). Haemosporidian parasite prevalence in *Culicoides* spp. in present study was 16.2%, in other studies it varies from 4.8% to 18.9% (Bernotienė et al., 2019; Žiegytė et al., 2021, 2022).

The third-most abundant species of this study, *C. kibunensis*, showed a high number of PCR-positive samples (10.4%). This species is distributed across Europe (Mathieu et al., 2012). Previous studies in Lithuania reported varying prevalence: 4.5% (Bernotienė et al., 2019), 45.5% in Vilnius and 3.0% in Neris Regional Park (Žiegytė et al., 2021), 7.8% in the Curonian Spit (Žiegytė et al., 2022). In Czech Republic, the prevalence in *C. kibunensis* pools was 51% (Synek et al., 2013). In our study, *C. kibunensis* not only had a high number of PCR-positive females but also had sporozoites of *H. belopolskyi* hHIICT1, *H. homominutus* hCUKI1, *H. parabelopolskyi* hSYAT01, hSYAT02, and *Haemoproteus* sp. hSYAT13 in salivary gland preparations (Table 3.4.1.1., Figure 3.4.1.2.), confirming likely involvement of *C. kibunensis* in natural transmission of these parasites. This biting midge species was already known to be a likely competent vector for *H. pallidus* hPFC1, *H. minutus* hTURDUS2, and *H. asymmetricus* hTUPHI01 (Bernotienė et al., 2019; Žiegytė et al., 2021). *Culicoides kibunensis*

demonstrates flexibility in vertebrate host selection, feeding on both mammals and birds (Martínez-de la Puente et al., 2015).

Culicoides festivipennis, another notable biting midge species in our study, has been reported to be positive for *Haemoproteus* DNA in several studies, including ours (Bernotienė et al., 2019; Bobeva et al., 2014; Synek et al., 2013; Žiegytė et al., 2022). Although sporozoites have never been observed in its salivary gland preparations, *C. festivipennis* might be a competent vector for *Haemoproteus* parasites, due to its ornithophilic feeding habits (Pettersson et al., 2013). The low prevalence of *Haemoproteus* infections in wild *Culicoides* (Bernotienė et al., 2019; Bernotienė and Valkiūnas, 2016; Bobeva et al., 2013, 2014; Žiegytė et al., 2021, 2022) indicates that more extensive sampling may be required to estimate involvement of *C. festivipennis* in transmission of these parasites. In present study, presence of sporozoites of *H. belopolskyi* hHIICT1 was confirmed in *C. festivipennis* salivary glands (Figure 3.4.1.2.a).

Although *C. segnis* was not among the most abundant species in our study, it exhibited a high PCR positivity for *Haemoproteus* and a notable prevalence of sporozoites in salivary gland preparations (Table 3.4.1.1.). The haemosporidian parasite prevalence determined by molecular methods in this research is similar to that previously reported in the Curonian Spit (Žiegytė et al., 2022). To date, *C. segnis* has been documented as positive for *Haemoproteus* DNA in Lithuania and the Czech Republic (Synek et al., 2013; Žiegytė et al., 2021, 2022), and it has recently been confirmed as a likely competent vector for *H. majoris* hCCF5 and *H. tartakovskyi* hHAWF1 (Žiegytė et al., 2022). Our study expands the list of possibly transmitted by *C. segnis* haemoproteids by adding four more *Haemoproteus* species and genetic lineages: *H. minutus* hTURDUS2, *H. asymmetricus* hTUPHI01, *H. fringillae* hCCF3, and *H. majoris* hPHSIB1.

Culicoides impunctatus, a commonly used experimental model for *Haemoproteus* infections (Bukauskaitė et al., 2019), was also collected and dissected in our study (Table 3.4.1.1.). This species has been shown to be a competent for completing sporogony insect for at least 13 *Haemoproteus* species (Bukauskaitė et al., 2019; Ilgūnas et al., 2019). However, all *C. impunctatus* samples in our study were PCR-negative for *Haemoproteus* DNA, consistent with literature reporting low or no prevalence of positive samples (Bernotienė and Valkiūnas, 2016; Žiegytė et al., 2021). This suggests that while *C. impunctatus* can theoretically transmit *Haemoproteus* parasites, natural transmission might be rare due to its mammophilic behavior (Blackwell et al., 1992). Although *Haemoproteus* DNA was detected in these

midges, indicating occasional feeding on birds, a second blood meal from a susceptible bird would be required to transmit parasites. Given that the sporozoites need about seven days to develop after an initial infected blood meal, this scenario is unlikely in natural conditions. *Culicoides impunctatus* is known to be bivoltine (having two generations per year) and autogenous (producing the first batch of eggs without a blood meal) (Blackwell et al., 1992; Mullen and Murphee, 2019). After laying their initial batch of eggs, females become responsive to animal bait and light, and a blood meal could lead to a second batch of eggs (Boorman and Goddard, 1970). However, a third batch is unlikely (though not impossible), and *C. impunctatus* might not feed on a vertebrate host again. Therefore, even if these midges are infected by *Haemoproteus* parasites, they might not transmit them. Limited studies on *C. impunctatus*'s biology (Blackwell et al., 1992; Boorman and Goddard, 1970) suggest that its lack of natural infections is likely due to a combination of factors: feeding habits, bivoltism, and autogeny.

Culicoides reconditus is reported here for the first time as both PCR-positive and capable of supporting *Haemoproteus* sporogonic development. Although this species is widespread in Europe (Mathieu et al., 2012), it is usually found in low numbers in Lithuania (Bernotienė et al., 2019; Chagas et al., 2022; Žiegytė et al., 2021). Nonetheless, it has been noted as a major *Culicoides* species present in blue tit *Cyanistes caeruleus* (Linnaeus, 1758) nest boxes in Germany (Garrido-Bautista et al., 2022). Despite its lower abundance in Lithuania, this study confirms its possible involvement in *Haemoproteus* transmission. Further research at various locations is needed to elucidate its feeding preferences, breeding sites, and role in *Haemoproteus* transmission.

Our interaction network analysis reveals the complex relationships between parasites and their vectors (Figures 3.4.1.3. and 3.4.1.4.). *Culicoides kibunensis* stands out for its likely significant role in *Haemoproteus* parasite transmission in Europe. It interacts with 23 *Haemoproteus* lineages, showing the highest diversity of *Haemoproteus* lineages among the biting midges in our study. This shows that *C. kibunensis* lacks specialization and might feed on many bird species. Notably, its primary interactions are with *H. minutus* hTURDUS2 and *H. asymmetricus* hTUPHI01, which are primarily associated with *Turdus merula* Linnaeus, 1758 and *Turdus philomelos* C.L.Brehm, 1831, respectively, though these lineages have been reported in other bird species as well.

Culicoides segnis also exhibited a high number of interactions with *Haemoproteus* lineages, totaling 16, with notable interactions with *H.*

asymmetricus hTUPHI01. This suggests that *C. segnis* may play a significant role in the transmission of certain *Haemoproteus* parasites, despite sporozoites only recently being identified in its salivary glands. It is likely that *C. segnis* has a broader host range in terms of feeding preferences. Additionally, *C. circumscriptus* was found to have a high number of interactions with various *Haemoproteus* species (11 in total). Although, in our study we managed to collect only three specimens this could be attributed to the fact that some *Culicoides* species prefer different habitat heights (Pettersson et al., 2013; Service, 1971) and *C. circumscriptus* in other studies were more frequently sampled at elevations of 20–26 meters above the ground (Braverman and Linley, 1993; Černý et al., 2011), whereas C. *kibunensis* was more commonly collected at ground level (Service, 1971), where Turdidae birds spend most of their time during the day.

The VU Kairėnai Botanical Garden emerged as the site with the highest abundance of both *Haemoproteus* lineages and *Culicoides* species. This may be attributed to its diverse microhabitats, including deciduous and coniferous trees, various bushes, both naturally occurring and planted vegetation, ponds, small rivers providing soil moisture, dead leaves, and nutrient-rich soil – ideal breeding conditions for *Culicoides*. Anthropogenic influence is also low (clean environment). The VU Kairėnai Botanical Garden had the highest number of parous females and detected *Culicoides* species, including *C. circumscriptus* (only found there), and higher numbers of *C. festivipennis*, *C. kibunensis*, *C. pallidicornis*, and *C. segnis*.

Our study identified 26 different *Haemoproteus* lineages, compared to a maximum of 11 reported in Lithuania (Bernotienė et al., 2019; Chagas et al., 2022; Žiegytė et al., 2021, 2022, 2023) and nine in Bulgaria (Bobeva et al, 2014). The Czech Republic study reported the lowest lineage diversity (five) (Synek et al., 2013), although the used methods might not be sensitive enough to determine co-infections and thus some parasite species and lineage diversity might be undetected. On the other hand, our observed variation might likely result from the diverse study sites with varying land use and anthropogenic influences, leading to higher bird species diversity and, consequently, greater parasite lineage diversity. Most studies also detected *Plasmodium* DNA in analyzed insects, indicating that the biting midges had fed on infected birds. However, since *Plasmodium* does not complete its sporogonic development in *Culicoides*, these insects are not considered vectors for *Plasmodium* parasites (Gutiérrez-López et al., 2016; Valkiūnas, 2005).

The VU Kairėnai Botanical Garden exhibited the highest diversity of *Haemoproteus* lineages, with 13 out of 26 being reported exclusively at this

site (Table 3.4.1.2.). This high diversity may be linked to specific host-parasite interactions. For instance, *Haemoproteus* sp. hSYAT13 and *H. homogeneae* hSYAT16 were previously reported only in *Sylvia atricapilla* (Linnaeus, 1758), while *H. homominutus* hCUKI1 was noted in *Turdus philomelos* and *Turdus viscivorus* Linnaeus, 1758 (Valkiūnas et al., 2019). This suggests that these bird species were more common in the VU Kairėnai Botanical Garden compared to other study sites. A recent study in Slovakia found *S. atricapilla* to be the most abundant bird species throughout the year, excluding winter (Šujanová et al., 2021), which may be true for Lithuania as well, although this has not been studied.

Conversely, some *Haemoproteus* lineages and species are found in a limited number of bird species belonging to certain families. For example, *H. majoris* hCWT4 and *H. magnus* hROFI1 were reported exclusively in Puvočiai village (Table 3.4.1.2.). Both lineages were reported in a wide range of bird species (21 in total), making it difficult to determine the specific host from which *Culicoides* acquired the infection (http://130.235.244.92/MalAvi/; accessed April 24, 2024). *Haemoproteus* sp. hCIRCUM05 was found only in Brinkiškės village and is known to infect only Corvidae species (http://130.235.244.92/MalAvi/; accessed April 24, 2024). Corvidae are successful in urban and anthropogenically influenced environments (Benmazouz et al., 2021), which may explain the presence of this parasite in Brinkiškės village. Similarly, *Haemoproteus syrnii* hCULKIB01, reported in a few Strigiformes species (http://130.235.244.92/MalAvi/; accessed April 24, 2024), was recorded only in Verkiai Regional Park, suggesting that this park may offer a more suitable habitat for Strigiformes compared to other sites.

Haemoproteus asymmetricus hTUPHI01 and *H. minutus* hTURDUS2 were found at all study sites and exhibited the highest prevalence (Tables 3.4.1.1. and 3.4.2.1.). These lineages are commonly reported in *Turdus philomelos* and *Turdus merula* (Valkiūnas, 2005; Valkiūnas et al., 2021), respectively, and their high prevalence across all sites may be due to the broad range of their hosts. *Turdus philomelos* and *T. merula* are abundant in Lithuania during spring and summer and autumn, with their populations showing an increasing trend (IUCN, 2023).

Our study identified nine co-infections by PCR methods. Co-infections are common in naturally infected birds (Clark et al., 2016; Himmel et al., 2020; Soares et al., 2016; Šujanová et al., 2021; Van Rooyen et al., 2013), and multiple parasite lineages can develop within a single *Culicoides* biting midge. However, the effects of co-infections on the vector are not well understood

(Carlson et al., 2018; Marchand, 2011), particularly in *Culicoides*. One co-infected specimen was also positive for sporozoites, but the currently used methodology does not allow for species-specific identification. The high prevalence of co-infections in *Sylvia atricapilla*, a species known for frequent co-infections (Santiago-Alarcon et al., 2013; Šujanová et al., 2021), may have influenced the observed prevalence of co-infections in *Culicoides*.

Remarkably, nearly 30% of PCR-positive Culicoides were also positive for Haemoproteus sporozoites (Table 3.4.1.1.). This represents the highest positivity reported using integrative approaches (dissection, microscopy, and molecular methods) (Bernotienė et al., 2019; Chagas et al., 2022; Žiegytė et al., 2021, 2022, 2023). The number of sporozoites varied, with some salivary gland preparations showing only a few sporozoites while others had many (Figure 3.4.1.2.r). This variability might be due to 1) the recent release of sporozoites from oocysts; 2) higher infection levels in *Culicoides* females, or 3) greater susceptibility of specific species or individuals. However, the lifespan of *Haemoproteus* sporozoites in *Culicoides* salivary glands and the number of sporozoites injected during a blood meal remain insufficiently investigated, and these factors may vary between parasite and vector species.

Table 3.4.2.1. *Haemoproteus* lineages found in researched areas: BG – VU Kairėnai Botanical Garden, P – Puvočiai and its surrounding areas, VRP – Verkiai Regional Park, and B – Brinkiškės and its surrounding areas.

Parasite species and Lineage	**BG**	**P**	**VRP**	**B**	**Total**
Haemoproteus asymmetricus hTUPHI01	40	9	42	4	95
H. belopolskyi hHIICT1	9	1	3	1	14
H. fringillae hCCF3		1	1		2
H. homogeneae hSYAT16	1				1
H. homominutus hCUKI1	2				2
H. magnus hROFI1		1			1
H. majoris hCCF5	2				2
H. majoris hCWT4		1			1
H. majoris hPARUS1		1	1		2
H. majoris hPHSIB1		1			1
H. majoris hWW2	1				1
H. minutus hTUCHR01			1		1
H. minutus hTURDUS2	14	5	12	3	34

H. parabelopolskyi hSYAT01	1		2		3
H. parabelopolskyi hSYAT02	2		3		5
H. syrnii hCULKIB01			1		1
Haemoproteus sp. hCCF2	1				1
Haemoproteus sp. hCIRCUM05				1	1
Haemoproteus sp. hCULKIB02	1				1
Haemoproteus sp. hCULKIB03	1				1
Haemoproteus sp. hCULKIB04	1				1
Haemoproteus sp. hCULKIB05	1				1
Haemoproteus sp. hCULPIC01	1				1
Haemoproteus sp. hCULPIC02	1				1
Haemoproteus sp. hHAWF6	1				1
Haemoproteus sp. hSYAT13	2				2
Plasmodium sp. hCULFES01	1				1
Plasmodium sp. hCULOBS01	1				1
P. vaughani hSYAT05	2				2
Co-infection	4	2	2	1	9
Total	**90**	**22**	**68**	**10**	**190**

Sporozoites of *Haemoproteus* species in *Culicoides* are similar, but differences have been reported, suggesting that morphological analysis could aid in parasite identification (Bukauskaitė et al., 2015). Sporozoites of *H. homominutus* hCUKI1 (Figure 3.4.1.2.b), *H. asymmetricus* hTUPHI01 (Figure 3.4.1.2.l – n), and *H. minutus* hTURDUS2 (Figure 3.4.1.2.o – q) appeared larger compared to others, such as *H. parabelopolskyi* (compare Figure 3.4.1.2.b with Figure 3.4.1.2.g – i). However, without detailed morphometric and statistical analysis, no definitive conclusions can be made. Future studies should address these issues, as the differences in sporozoite numbers among infected *Culicoides* did not permit such analysis.

While several parasite lineages were detected, the mechanisms influencing parasite transmission in nature remain incompletely understood. However, it is evident that *C. kibunensis*, *C. pictipennis*, and *C. segnis* are

likely significant *Haemoproteus* vectors in Lithuania due to their abundance and ornithophilic habits (Bobeva et al., 2015; González et al., 2022; Tomazatos et al., 2020). Future studies should further investigate the biology of hosts, parasites, and vectors to better understand their roles in transmission.

Moreover, the transmission of vector-borne diseases requires the simultaneous presence of an infected host, a competent vector, and a susceptible host in the same location and timeframe. And also abiotic factors. It is important to note that all parasites identified in this study infect birds (Table 3.4.1.1.) (Valkiūnas and Iezhova, 2022). This allowed us to assess that all *Culicoides* PCR-positive for *Plasmodium/Haemoproteus*, even if sporozoites were not present in their salivary glands, were feeding on birds' blood. This information is valuable given the limited research on *Culicoides* feeding preferences.

We also detected avian *Plasmodium* DNA in one sample, despite *Culicoides* not transmitting these parasites. This finding, consistent with previous reports (Bernotienė et al., 2019; Bernotienė and Valkiūnas, 2016; Bobeva et al., 2014; Ferraguti et al., 2013b), indicates that the biting midge fed on an infected bird. However, since *Plasmodium* does not complete its development in *Culicoides*, these insects cannot be considered vectors for *Plasmodium* parasites.

CONCLUSIONS

1. Mosquito larvae were present in water bodies from March until September, with the highest species diversity and abundance observed in April and May in Lithuania. Mosquito species diversity and abundance of certain species was statistically significantly related to temporality of water body, bottom cover, pH, amount of nitrates in water bodies and seasonality.
2. *Coquillettidia richiardii*, *Culex pipiens*, and species of genus *Culiseta* had statistically significantly higher prevalences of haemosporidian parasites, making them important objects in haemosporidian parasite research. *Ochlerotatus* mosquitoes were the least infected. The highest prevalence in all mosquitoes was observed in July and August. Five *Plasmodium* and three *Haemoproteus* species as well as various combinations of *Plasmodium*, *Haemoproteus*, and *Leucocytozoon* co-infections were detected in mosquitoes.
3. *Culex pipiens* supports complete sporogony of *Plasmodium matutinum* (genetic pLINN1) and thus is a likely natural vector.
4. Three *Trypanosoma* species were detected in active *Culex pipiens* mosquitoes. Hibernating *C. pipiens* mosquitoes were infected only with monoxenous trypanosomatids and were not infected with haemosporidian parasites. This indicates the non-essential role of hibernating *C. pipiens* mosquitoes as reservoirs of these parasites.
5. Sporozoites of *Haemoproteus* parasites (genetic lineages hCUKI1, hCULPIC02, hROFI1, hSYAT01, hSYAT13, hSYAT16) were found in salivary glands of biting midges for the first time. *Haemoproteus* sporozoites belonging to five genetic lineages were detected in salivary glands of *C. kibunensis* (lineages hCUKI1, hHIICT1, hSYAT01, hSYAT13, hSYAT16), five in *C. pictipennis* (lineages hSYAT01, hSYAT13, hSYAT16, hTUPHI01, hTURDUS2), four in *C. segnis* (lineages hCCF3, hPHSIB1, hTUPHI01, hTURDUS2), and one in *C. festivipennis* (lineage hHIICT1) for the first time. These biting midges are likely natural vectors of previously mentioned parasites.
6. Eight out of 13 *Culicoides* species were found to harbor DNA of 11 *Haemoproteus* and *Plasmodium* species (29 genetic lineages), this way showing the ornitophilic feeding behaviour of these *Culicoides* species.

7. *Culicoides reconditus* supports complete sporogony of *Haemoproteus* parasites (*H. magnus* hROFI1) and thus is a likely natural vector of these pathogens.

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Appendix

Supplementary Table 1. Coordinates with collection place of water bodies where mosquito larvae were collected.

	Site	Coordinates
1.	Alytus city	54°23'19.9"N 24°04'22.3"E
2.	Alytus city	54°23'24.7"N 24°03'47.4"E
3.	Alytus city	54°23'26.6"N 24°04'39.0"E
4.	Alytus city	54°23'27.9"N 24°04'41.1"E
5.	Alytus city	54°24'09.3"N 24°01'34.5"E
6.	Alytus city	54°24'14.8"N 24°01'30.5"E
7.	Alytus city	54°24'34.6"N 24°00'14.0"E
8.	Alytus municipality	54°24'53.9"N 23°59'04.0"E
9.	Elektrėnai municipality	54°45'25.8"N 24°47'59.1"E
10.	Elektrėnai municipality	54°45'28.1"N 24°48'04.7"E
11.	Elektrėnai municipality	54°45'29.8"N 24°48'05.7"E
12.	Elektrėnai municipality	54°45'36.5"N 24°48'19.8"E
13.	Elektrėnai municipality	54°45'37.4"N 24°48'23.5"E
14.	Elektrėnai municipality	54°45'42.7"N 24°48'35.8"E
15.	Elektrėnai municipality	54°46'04.4"N 24°49'36.5"E
16.	Elektrėnai municipality	54°46'04.7"N 24°49'36.5"E
17.	Elektrėnai municipality	54°46'07.3"N 24°49'26.9"E
18.	Elektrėnai municipality	54°46'12.0"N 24°49'27.7"E
19.	Elektrėnai municipality	54°46'43.6"N 24°48'40.3"E
20.	Elektrėnai municipality	54°47'03.4"N 24°48'34.6"E
21.	Elektrėnai municipality	54°47'46.6"N 24°50'59.6"E
22.	Elektrėnai municipality	54°47'47.7"N 24°50'57.8"E
23.	Elektrėnai municipality	54°47'53.2"N 24°50'58.3"E
24.	Elektrėnai municipality	54°47'59.0"N 24°51'12.0"E
25.	Elektrėnai municipality	54°48'00.7"N 24°51'20.5"E
26.	Elektrėnai municipality	54°48'09.2"N 24°47'41.3"E
27.	Elektrėnai municipality	54°48'09.4"N 24°47'45.4"E
28.	Elektrėnai municipality	54°48'12.5"N 24°47'43.9"E
29.	Elektrėnai municipality	54°48'13.6"N 24°46'44.5"E
30.	Elektrėnai municipality	54°48'13.9"N 24°46'41.6"E

31.	Elektrėnai municipality	54°48'14.9"N 24°46'43.6"E
32.	Elektrėnai municipality	54°48'15.9"N 24°46'39.9"E
33.	Elektrėnai municipality	54°48'18.1"N 24°46'48.2"E
34.	Elektrėnai municipality	54°48'18.8"N 24°46'48.4"E
35.	Elektrėnai municipality	54°48'19.9"N 24°46'49.3"E
36.	Elektrėnai municipality	54°48'20.8"N 24°46'50.9"E
37.	Elektrėnai municipality	54°48'21.2"N 24°47'14.6"E
38.	Elektrėnai municipality	54°48'21.4"N 24°47'15.1"E
39.	Elektrėnai municipality	54°48'21.8"N 24°47'13.4"E
40.	Elektrėnai municipality	54°48'26.0"N 24°46'54.2"E
41.	Elektrėnai municipality	54°48'47.0"N 24°50'55.5"E
42.	Elektrėnai municipality	54°48'47.9"N 24°49'42.7"E
43.	Elektrėnai municipality	54°48'57.9"N 24°49'56.7"E
44.	Elektrėnai municipality	54°48'58.0"N 24°49'51.1"E
45.	Elektrėnai municipality	54°48'58.2"N 24°49'55.1"E
46.	Elektrėnai municipality	54°49'00.2"N 24°50'34.4"E
47.	Elektrėnai municipality	54°49'19.5"N 24°47'13.8"E
48.	Prienai municipality	54°29'54.7"N 23°55'31.6"E
49.	Prienai municipality	54°29'54.8"N 23°55'20.0"E
50.	Prienai municipality	54°30'01.1"N 23°56'22.7"E
51.	Prienai municipality	54°30'02.1"N 23°55'45.1"E
52.	Prienai municipality	54°30'02.6"N 23°55'48.5"E
53.	Prienai municipality	54°30'17.1"N 23°55'22.2"E
54.	Prienai municipality	54°30'22.9"N 23°55'39.5"E
55.	Prienai municipality	54°30'28.9"N 23°55'16.3"E
56.	Prienai municipality	54°30'33.0"N 23°55'14.8"E
57.	Šiauliai city	55°56'54.9"N 23°16'02.4"E
58.	Šiauliai city	55°56'57.4"N 23°15'57.1"E
59.	Vilnius city	54°41'46.3"N 25°18'09.7"E
60.	Vilnius city	54°42'23.7"N 25°16'10.7"E
61.	Vilnius city	54°42'24.6"N 25°16'14.5"E
62.	Vilnius city	54°42'28.6"N 25°16'15.3"E
63.	Vilnius city	54°42'33.3"N 25°16'16.1"E
64.	Vilnius city	54°42'39.7"N 25°16'21.5"E

65.	Vilnius city (Belmontas)	54°41'09.0"N 25°21'29.6"E
66.	Vilnius city (Belmontas)	54°41'11.5"N 25°21'27.0"E
67.	Vilnius city (Belmontas)	54°41'15.4"N 25°21'27.9"E
68.	Vilnius city (Belmontas)	54°41'15.9"N 25°21'30.4"E
69.	Vilnius city (Belmontas)	54°41'16.7"N 25°21'27.4"E
70.	Vilnius city (Belmontas)	54°41'18.8"N 25°22'01.5"E
71.	Vilnius city (Belmontas)	54°41'19.0"N 25°21'31.1"E
72.	Vilnius city (Belmontas)	54°41'19.1"N 25°21'25.4"E
73.	Vilnius city (Belmontas)	54°41'19.1"N 25°21'31.1"E
74.	Vilnius city (Belmontas)	54°41'19.4"N 25°21'49.1"E
75.	Vilnius city (Belmontas)	54°41'19.5"N 25°21'49.1"E
76.	Vilnius city (Dvarčionys)	54°43'23.4"N 25°22'24.5"E
77.	Vilnius city (Dvarčionys)	54°43'23.7"N 25°22'24.1"E
78.	Vilnius city (Dvarčionys)	54°43'23.8"N 25°22'24.1"E
79.	Vilnius city (Dvarčionys)	54°43'24.9"N 25°22'26.5"E
80.	Vilnius city (Dvarčionys)	54°43'25.4"N 25°22'24.7"E
81.	Vilnius city (Dvarčionys)	54°43'26.3"N 25°22'25.5"E
82.	Vilnius city (Dvarčionys)	54°43'27.0"N 25°22'23.8"E
83.	Vilnius city (Dvarčionys)	54°44'02.7"N 25°22'32.4"E
84.	Vilnius city (Dvarčionys)	54°44'07.9"N 25°22'45.0"E
85.	Vilnius city (Dvarčionys)	54°44'09.7"N 25°22'45.7"E
86.	Vilnius city (Dvarčionys)	54°44'12.6"N 25°23'01.6"E
87.	Vilnius city (Dvarčionys)	54°44'13.1"N 25°23'00.1"E
88.	Vilnius city (Dvarčionys)	54°44'16.4"N 25°23'00.8"E
89.	Vilnius city (Dvarčionys)	54°44'19.3"N 25°23'01.5"E
90.	Vilnius city (Dvarčionys)	54°44'19.3"N 25°23'02.0"E
91.	Vilnius city (Dvarčionys)	54°44'19.4"N 25°23'02.0"E
92.	Vilnius city (Green Lakes)	54°45'41.3"N 25°18'44.1"E
93.	Vilnius city (Green Lakes)	54°45'48.8"N 25°18'47.2"E
94.	Vilnius city (Green Lakes)	54°47'47.8"N 25°19'22.5"E
95.	Vilnius city (Green Lakes)	54°47'48.8"N 25°18'48.2"E
96.	Vilnius city (Green Lakes)	54°47'51.5"N 25°19'04.3"E
97.	Vilnius city (Karoliniškės)	54°41'51.1"N 25°13'58.2"E
98.	Vilnius city (Karoliniškės)	54°41'51.2"N 25°13'58.6"E

99.	Vilnius city (Pilaitė)	54°41'38.1"N 25°09'35.6"E
100.	Vilnius city (Pilaitė)	54°41'48.2"N 25°10'03.7"E
101.	Vilnius city (Pilaitė)	54°42'01.4"N 25°09'35.7"E
102.	Vilnius city (Pilaitė)	54°42'05.7"N 25°09'42.3"E
103.	Vilnius city (Pilaitė)	54°42'08.9"N 25°09'46.8"E
104.	Vilnius city (Pilaitė)	54°42'20.7"N 25°09'54.2"E
105.	Vilnius city (Pilaitė)	54°42'23.7"N 25°11'39.4"E
106.	Vilnius city (Turniškės)	54°44'54.1"N 25°18'13.5"E
107.	Vilnius city (Turniškės)	54°44'59.3"N 25°18'32.3"E
108.	Vilnius city (Verkiai)	54°44'53.3"N 25°17'20.4"E
109.	Vilnius municipality	54°47'50.0"N 25°03'41.8"E
110.	Vilnius municipality	54°47'50.4"N 25°03'41.7"E
111.	Vilnius municipality	54°47'50.9"N 25°03'43.0"E
112.	Vilnius municipality	54°47'51.0"N 25°03'41.4"E
113.	Vilnius municipality	54°47'51.1"N 25°03'41.9"E
114.	Vilnius municipality	54°47'51.2"N 25°03'44.2"E
115.	Vilnius municipality	54°47'51.3"N 25°03'32.4"E
116.	Vilnius municipality	54°47'51.8"N 25°03'44.5"E
117.	Vilnius municipality	54°47'51.9"N 25°03'40.5"E
118.	Vilnius municipality	54°47'52.5"N 25°03'26.6"E
119.	Vilnius municipality	54°47'52.6"N 25°03'40.3"E
120.	Vilnius municipality	54°47'52.8"N 25°03'26.5"E
121.	Vilnius municipality	54°47'52.9"N 25°03'23.9"E
122.	Vilnius municipality	54°47'53.0"N 25°03'35.9"E
123.	Vilnius municipality	54°47'53.6"N 25°03'35.2"E
124.	Vilnius municipality	54°47'53.7"N 25°03'39.1"E
125.	Vilnius municipality	54°47'54.5"N 25°03'36.7"E
126.	Vilnius municipality	54°47'54.7"N 25°03'35.3"E
127.	Vilnius municipality	54°47'54.7"N 25°03'40.9"E
128.	Vilnius municipality	54°47'55.8"N 25°03'34.7"E
129.	Vilnius municipality	54°47'58.5"N 25°03'29.9"E
130.	Vilnius municipality	54°48'07.0"N 25°04'05.9"E
131.	Vilnius municipality	54°48'07.1"N 25°04'04.9"E
132.	Vilnius municipality	54°48'07.1"N 25°04'04.9"E

133.	Vilnius municipality	54°48'12.9"N 25°04'56.0"E
134.	Vilnius municipality	54°48'13.2"N 25°05'28.5"E

Supplementary Table 2. Experimental and molecular studies showing what haemosporidian parasite DNA or parasites were found in *Culex pipiens* mosquitoes. Information acquired from MalAvi database (accessed on June 17, 2024) and (Santiago-Alarcon et al., 2012; Nourani et al., 2020); ED – experimental data; PNV – possible natural vector.

Parasite species	Lineages	ED	PNV	Developmental stages found in mosquito	PCR	Tested part	Country/ References
Haemoproteus asymmetricus	hTUPHI01	–	–	–	+	whole mosquito	Lithuania (Valavičiūtė-Pocienė et al., 2024)
H. brachiatus	hLK03	–	–	–	+	whole mosquito	Lithuania (Valavičiūtė-Pocienė et al., 2024)
	hTURDUS2	–	–	–	+	abdomen with blood	Italy (Martínez-de la Puente et al., 2015)
					+	thorax, unfed	Czech Republic (Synek et al., 2013)
H. palloris	hWW1	–	–	–	+	head and thorax	Madagascar (Schmid et al., 2017)
H. passeris	hPADOM05	–	–	–	+	head and thorax	Turkey (Inci et al., 2012)
Haemoproteus sp.	hCIRCUM05h CXPIP28 hPADOM03	–	–	–	+	abdomen with blood	Italy (Martínez-de la Puente et al., 2015)
	hCOCOR15	–	–	–	+	whole mosquito	Japan (Shirotani et al., 2009)
	hCXPIP16 hCXPIP17 hCXPIP18 hCXPIP19	–	–	–	+	abdomen with blood	Japan (Ejiri et al., 2011a)
	hCXPIP19	–	–	–	+	abdomen	Japan (Odagawa et al., 2022)
		–	–	–	+	whole mosquito	Japan (Shirotani et al., 2009)
	hCXPIP27 hGAGLA03	–	–	–	+	whole mosquito	France (Zélé et al., 2014)
	hCXPIP29	–	–	–	+	head and thorax	Madagascar (Schmid et al., 2017)

Parasite species	Lineages	ED	PNV	Developmental stages found in mosquito	PCR	Tested part	Country/ References
Leucocytozoon sp.	lTUPHI05	–	–	–	+	whole mosquito	Austria (Schoener et al., 2017)
Plasmodium cathemerium	pPADOM02	–	–	–	+	abdomen with blood	Japan (Kim et al., 2009; Inumaru et al., 2021)
P. cathemerium	pPADOM02	–	–	–	+	whole mosquito	Japan (Ejiri et al., 2009; Shirotani et al., 2009; Kim et al., 2009)
		–	–	–	+	thorax	Japan (Kim and Tsuda, 2010; 2012; Ejiri et al., 2011a), Switzerlan(Glaizot et al., 2012; Lalin et al., 201
		–	–	–	+	thorax and head/ abdomen	Japan (Odagawa et al., 2022)
		–	–	–	+	whole mosquito	USA (Kimura et al., 2010)
	–	–	–	oocyst	–	–	USA (Huff, 1927; Herman, 1938)
P. circumflexum	pTURDUS1	–	–	–	+	thorax	Switzerland (Glaizot et al., 2012)
P. durae	–	–	–	sporozoite	–	–	Africa (Valkiūnas, 2005)
P. elongatum	pGRW06	–	–	–	+	whole mosquito	France (Zélé et al., 2014)
		–	–	–	+	–	Austria (Schoener et al., 2017; 2019)
	–	+	+	–	–	–	USA (Huff, 1927), Italy (Raffaele, 1934; Micks, 1949)

Parasite species	Lineages	ED	PNV	Developmental stages found in mosquito	PCR	Tested part	Country/ References
P. gallinaceum	pGALLUS01	–	–	–	+	thorax	Japan (Kim and Tsuda, 2010; 2012)
		–	–	–	+	whole mosquito	Japan (Shirotani et al., 2009)
P. garnhami	–	+	–	sporozoites	–	–	Egypt (Garnham, 1966)
P. giovannolai	–	+	–	sporozoites	–	–	Italy (Corradetti et al., 1963a,b)
P. homonucleophilum	pSW2	–	–	–	+	thorax	Switzerland (Glaizot et al., 2012)
		–	–	–	+	abdomen	Lithuania (Valavičiūtė-Pocienė et al., 2024)
P. juxtanucleare	pGALLUS02	–	–	–	+	thorax	Japan (Kim and Tsuda, 2012)
	–	+	–	sporozoites	–	–	Japan (Akiba, 1959)
P. kempi	–	+	–	sporozoites	–	–	USA (Christensen et al., 1983)
P. lophurae	–	–	–	oocyst	–	–	USA (Coggeshall, 1940)
P. matutinum	pLINN1	–	–	–	+	whole mosquito	France (Zélé et al., 2014), USA (Kimura et al., 2010)
		–	–	–	+	abdomen with blood	Italy (Martínez-de la Puente et al., 2015)
		–	–	–	+	head and thorax	Spain (Ferraguti et al., 2013b)
		–	–	–	+	–	Austria (Schoener et al., 2017)
		–	+	sporozoites	+	whole mosquito	Lithuania (Valavičiūtė-Pocienė et al., 2024)

Parasite species	Lineages	ED	PNV	Developmental stages found in mosquito	PCR	Tested part	Country/ References
P. matutinum	–	–	–	oocyst	–	–	USA (Huff, 1937; Manwell, 1940; 1947)
P. relictum	pGRW04	–	–	oocyst, sporozoites	+	–	Japan (Kim and Tsuda, 2015)
		–	–	–	+	thorax	Japan (Kim and Tsuda, 2012)
		–	–	–	+	head and thorax	Madagascar (Schmid et al., 2017)
		–	–	–	+	abdomen	Japan (Odagawa et al., 2022)
		+	–	sporozoites	+	–	Lithuania (Valkiūnas et al., 2015)
	pGRW11	–	–	–	+	abdomen with blood	Italy (Martínez-de la Puente et al., 2015), Japan (Kim et al., 2009)
		–	–	–	+	thorax	Japan (Kim and Tsuda, 2010), Switzerland (Glaizot et al., 2012; Lalubin et al., 2013)
		–	–	oocyst, sporozoites	+	–	Japan (Kim and Tsuda, 2015)
		+	–	–	–	–	Lithuania (Kazlauskienė et al., 2013)
		–	–	–	+	whole mosquito	France (Zélé et al., 2014)
	pSGS1	–	–	–	+	–	Romania (Ionică et al., 2017), Japan (Kim and Tsuda, 2015), Austria (Schoener et al., 2019)
		–	–	–	+	whole mosquito	France (Zélé et al., 2014),

Parasite species	Lineages	ED	PNV	Developmental stages found in mosquito	PCR	Tested part	Country/ References
P. relictum	pSGS1	–	–	–	+	whole mosquito	Japan (Kim et al., 2009), Portugal (Ventim et al., 2012)
		–	–	–	+	abdomen with blood	Italy (Martínez-de la Puente et al., 2015)
		–	–	–	+	thorax	Japan (Kim and Tsuda, 2010), Switzerland (Glaizot et al., 2012)
		–	–	oocyst, sporozoites	+	–	Japan (Ejiri et al., 2011a)
		–	–	–	+	thorax and head/ abdomen	Japan (Odagawa et al., 2022)
		+	–	–	–	–	Lithuania (Kazlauskienė et al., 2013)
		–	–	–	+	head and thorax	Spain (Ferraguti et al., 2013b), Turkey (Inci et al., 2012), Switzerland (Lalubin et al., 2013), Austria (Schoener et al., 2017)
	–	–	–	–	+	–	Romania (Ionică et al., 2017)
	–	+	–	sporozoites	–	–	Germany (Ruge, 1901; Neumann, 1908), Algeria (Sergent and Sergent, 1907),
	–	+	–	sporozoites	–	–	USA (Tate and Vincent, 1934), Columbia (Hunninen, 1951; 1953)
P. rouxi	–	–	+	sporozoites	–	–	USA (Manwell, 1947; Huff, 1932)

Parasite species	Lineages	ED	PNV	Developmental stages found in mosquito	PCR	Tested part	Country/ References
Plasmodium sp.	pAFTRU5	–	–	–	+	head and thorax	Switzerland (Lalubin et al., 2013)
		–	–	–	+	abdomen with blood	Italy (Martínez-de la Puente et al., 2015)
	pCOLL1	–	–	–	+	whole mosquito	France (Zélé et al., 2014)
		–	–	–	+	head and thorax	Switzerland (Lalubin et al., 2013), Spain (Ferraguti et al., 2013b)
	pCXPIP01 pCXPIP02 pCXPIP03 pCXPIP04 pCXPIP05 pCXPIP06 pCXPIP07	–	–	–	+	whole mosquito	USA (Kimura et al., 2010)
	pCXPIP09	–	–	–	+	whole mosquito	Japan (Ejiri et al., 2009; Shirotani et al., 2009; Kim et al., 2009)
		–	–	–	+	abdomen with blood	Japan (Kim et al., 2009; Inumaru et al., 2021)
		–	–	–	+	thorax	Japan (Kim and Tsuda, 2010; 2012)
		–	–	oocyst, sporozoites	+	–	Japan (Kim and Tsuda, 2015)
		–	–	–	+	thorax, mosquito with blood	Japan (Ejiri et al., 2011a)
		–	–	–	+	head and thorax/abdomen	Japan (Odagawa et al., 2022)
	pCXPIP10 pCXPIP11 pCXPIP12	–	–	–	+	whole mosquito	Japan (Kim et al., 2009)

Parasite species	Lineages	ED	PNV	Developmental stages found in mosquito	PCR	Tested part	Country/ References
Plasmodium sp.	pCXPIP13 pCXPIP14	–	–	–	+	whole mosquito	Japan (Kim et al., 2009)
	pCXPIP10 pCXPIP11 pCXPIP12 pCXPIP13 pCXPIP14	–	–	–	+	thorax	Japan (Kim and Tsuda, 2010; 2012)
	pCXPIP15	–	–	–	+	abdomen with blood	Japan (Ejiri et al., 2011a)
	pCXPIP20 pCXPIP21 pCXPIP22 pCXPIP23	–	–	–	+	head and thorax	Turkey (Inci et al., 2012)
	pCXPIP24 pCXPIP25 pCXPIP26	–	–	–	+	whole mosquito	France (Zélé et al., 2014)
	pCXPIP30	–	–	–	+	head and thorax	Madagascar (Schmid et al., 2017)
	pCXPIP31	–	–	–	+	thorax, unfed	Japan (Ejiri et al., 2011b)
	pCXPIP32 pCXPIP33	–	–	–	–	–	Italy (Iurescia et al unpubl)
	pCXQUI01	–	–	–	+	thorax, unfed	Japan (Kim and Tsuda, 2012)
	pDELURB4	–	–	–	+	abdomen with blood	Italy (Martínez-de la Puente et al., 2015)
		–	–	–	+	whole mosquito	France (Zélé et al., 2014), Austria (Schoener et al., 2017)
	pDELURB5	–	–	–	+	–	Austria (Schoener et al., 2017)
		–	–	–	+	whole mosquito	France (Zélé et al., 2014)
	pDONANA03 pDONANA05	–	–	–	+	whole mosquito	Austria (Schoener et al., 2017), France (Zélé et al., 2014)

Parasite species	Lineages	ED	PNV	Developmental stages found in mosquito	PCR	Tested part	Country/ References
Plasmodium sp.	pPADOM01	–	–	–	+	whole mosquito	France (Zélé et al., 2014)
		–	–	–	+	head and thorax	Switzerland (Lalubin et al., 2013)
	pSPHUM05	–	–	–	+	thorax, mosquito with blood	Japan (Inumaru et al., 2021)
	pSPMAG10 pZOCAP03	–	–	–	+	abdomen	Japan (Odagawa et al., 2022)
	pSYCON02	–	–	–	+	head and thorax	Japan (Odagawa et al., 2022)
P. subpraecox	–	+	–	sporozoites	–	–	Italy (Raffaele, 1932)
P. unalis	pTUMIG03	–	–	–	+	whole mosquito	USA (Kimura et al., 2010)
P. vaughani	pSYAT05	–	–	–	+	whole mosquito	France (Zélé et al., 2014), USA (Kimura et al., 2010), Lithuania (Valavičiūtė-Pocienė et al., 2024)
P. vaughani	pSYAT05	–	–	–	+	abdomen with blood	Italy (Martínez-de la Puente et al., 2015)
		–	–	–	+	thorax	Japan (Kim and Tsuda, 2010), Switzerland (Glaizot et al., 2012)
		–	–	–	+	head and thorax	Switzerland (Lalubin et al., 2013), Turkey (Inci et al., 2012)
		–	–	–	+	–	Austria (Schoener et al., 2017; Raffaele, 1934)
		–	–	–	–	thorax, unfed	Czech Republic (Synek et al., 2013)

Parasite species	Lineages	ED	PNV	Developmental stages found in mosquito	PCR	Tested part	Country/ References
P. vaughani	–	+	–	sporozoites	–	–	Italy (Corradetti and Scanga, 1972)

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Supplementary Table 3. *Trypanosoma* species found in *Culex* mosquitoes in various studies. ER – experimental research; WM – wild-caught mosquitoes.

Trypanosomatid species	Isolate	Origin of isolate	ER	WM	Country, source
Trypanosoma thomasbancrofti	CUL15 CUL98	*Culex pipiens*	+		Czech Republic (Fialová et al., 2021; Votypka et al., 2012)
	OF19	*Ornithomya fringilline**	+		Czech Republic (Fialová et al., 2021)
	PAS343	*Phylloscopus sibilatrix**	+		Czech Republic (Fialová et al., 2021)
T. theileri	-	*C. pipiens*		+	Czech Republic (Fialová et al., 2021; Brotánková et al., 2022)
T. culicavium	CUL1 CUL6 CUL24 CUL28 CUL31	*C. pipiens*		+	Czech Republic (Votypka et al., 2012)
	-	*C. pipiens s.l./ torrentium, Culex modestus, Culex* spp		+	Austria (Schoener et al., 2018)
Trypanosoma sp.	CUL5 CUL2	*C. pipiens*		+	Czech Republic (Zídková et al., 2012)
T. avium		*C. pipiens s.l./ torrentium*		+	Austria (Schoener et al., 2018)
		C. pipiens, Culex tarsalis		+	USA (Van Dyken, 2006)

*- *Culex* mosquitoes were experimentally infected using these isolates.

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SANTRAUKA

ĮVADAS

Kraują siurbiantys vabzdžiai ekosistemose ir kasdieniame žmonių gyvenime atlieka ypatingai svarbų vaidmenį. Jie ne tik kelia nepatogumus maitindamiesi krauju, bet ir geba perduoti patogenus, sukeliančius ligas ir nužudančius šimtus tūkstančių žmonių kasmet. Dvisparniai (Diptera) yra vienas evoliuciškai pažangiausių (Wiegmann ir kt., 2011), antras pagal apibūdintų rūšių skaičių (Yeates ir Wiegmann, 2005), bei pirmas pagal kraujasiurbių rūšių įvairovę vabzdžių būrys (Lehane, 2005). Diptera būrys apima tokias gerai žinomas šeimas kaip Culicidae (tikrieji uodai), Ceratopogonidae (mašalai), Simuliidae (upiniai mašalai), Tabanidae (sparvos), Hippoboscidae (briedmusės), Psychodidae (kandiniai uodeliai), Muscidae (musės) (Lehane, 2005). Vien Culicidae šeimoje yra daugiau nei 3,7 tūkstančiai rūšių (Harbach, 2024). Keturios Ceratopogonidae šeimai priklausančios gentys, kuriose yra kraujasiurbių vabzdžių rūšių – *Forcipomyia, Leptoconops, Austroconops*, bet didžiausia – *Culicoides* su daugiau nei 1,4 tūkstančiais rūšių (Borkent ir Dominiak, 2020).

Vektorių tyrimai yra svarbūs siekiant geriau suprasti parazitų rūšių plitimą gamtoje, taip pat numatyti ligų plitimo dėl klimato kaitos galimybes ir apsaugoti nykstančias rūšis. Tiek tikrieji uodai, tiek smulkieji mašalai gali pernešti parazitinius Haemosporida ir Trypanosomatida būriams priklausančius pirmuonis, kurie aptinkami visame pasaulyje ir Lietuvoje dažnai aptinkami paukščiuose. Haemosporida būriui priklausančios parazitų rūšys gali užkrėsti įvairius gyvūnus – varliagyvius, roplius, paukščius, žinduolius (Valkiūnas, 2005). Paukščių hemosporidiniai parazitai yra plačiai paplitę ir gali sukelti sunkias ligas ar net mirtį, ypatingai paukščiams, kurie nėra prisitaikę prie konkrečių parazitų rūšių (Garnham, 1966; Ilgūnas ir kt., 2016). Ilgą laiką paukščių maliarijos tyrimai naudoti kaip modeliniai žmogaus maliarijos tyrimams. Anksčiau šie tyrimai padėjo išsiaiškinti hemosporidinių parazitų gyvenimo ciklą, sukurti *in vitro* auginimo technikas, patobulinti chemoterapiją bei prisidėjo prie daugelio kitų medicininės parazitologijos aspektų (Valkiūnas, 2005).

Paukščių kraujo parazitų, jų vektorių ir perdavimo dinamikos tyrimų ir informacijos apie juos trūksta. Europoje tik 6-iose smulkiųjų mašalų rūšyse buvo aptikti hemosporidinių parazitų sporozoitai (Žiegytė ir kt., 2023), o 9-ios tikrųjų uodų rūšys eksperimentiškai buvo patvirtintos kaip šių parazitų pernešėjai (Ferreira ir kt., 2020; Santiago-Alarcon ir kt., 2012).

Tripanosomatidai, ypač 1843 m. David Gruby aprašyta gentis *Trypanosoma*, yra gerai žinomi parazitai dėl sunkių ligų, kurias jie sukelia žmonėms ir naminiams gyvūnams (Zídková ir kt., 2012). Nors šiame kontekste jos buvo plačiai tiriamos, tačiau laukinėje gamtoje, ypač paukščius parazituojančios, tripanosomos sulaukė mažiau dėmesio, nepaisant įrodymų, kad kai kurios rūšys yra patogeniškos. Paukščių tripanosomas perduoda vabzdžiai vektoriai, priklausantys šeimoms Hippoboscidae, Simuliidae, Culicidae ir Ceratopogonidae (Bernotienė ir kt., 2020; Molyneux, 1977; Votýpka ir Svobodová, 2004). Duomenų apie tikruosiuose uoduose ir smulkiuosiuose mašaluose aptiktus *Trypanosoma* parazitinius žiuželinius pirmuonis yra nedaug (13-oje tikrųjų uodų ir 7-iose smulkiųjų mašalų rūšyse) (Brotánková ir kt., 2022; Kazak ir kt., 2023; Miltgen ir Landau, 1982; Molyneux, 1977; Votypka ir kt., 2002; 2012).

Kraują siurbiančių vabzdžių tyrimai yra labai svarbūs ne tik ligų prevencijai, bet ir jų vaidmens ekologijoje bei jų elgsenos supratimui. Vabzdžių tyrimai padeda identifikuoti potencialius patogenų pernešėjus. Kai kurios kraujasiurbių vabzdžių rūšys geba maitintis skirtingų šeimininkų krauju ir tokiu būdu iš vienos šeimininko rūšies gali perduoti ligas netipiniams kitų rūšių šeimininkams, taip tapdami „jungiančiaisiais vektoriais“ (angl. – bridge vectors) (Truc ir kt., 2013). Mūsų pasauliui tampant vis labiau kosmopolitišku, rizikos, susijusios su šiais vektoriais, auga. Jų ekologijos, gyvenimo ciklų bei mitybos prioritetų suvokimas yra būtinas norint sukurti veiksmingas kontrolės priemones, ypač tose vietose, kur šie vabzdžiai yra endeminiai arba kur įsikuria invazinės rūšys. Kitas svarbus aspektas yra klimato kaita, kuri gali stipriai paveikti invazinių rūšių ir jų perduodamų parazitų paplitimą. Didėjant temperatūrai ir keičiantis oro sąlygoms, daugelio kraują siurbiančių vabzdžių arealai slenka į naujus regionus, o taip didėja rizika plisti ligoms į anksčiau nepaveiktas sritis.

Nors apie kraują siurbiančių vabzdžių medicininį ir ekonominį poveikį žinoma daug, tačiau vis dar lieka spragų mūsų turimose žiniose: apie jų nesubrendusias stadijas, tam tikrus elgsenos modelius. Reikalingi tolimesni tyrimai, kad būtų ištirta jų galimybė platinti egzistuojančius ir naujai atsirandančius vabzdžių pernešamus ligų sukėlėjus, bei vabzdžių gebėjimą prisitaikyti prie kintančių aplinkos sąlygų. Apibendrinant – nors kraujo parazitų tyrimai yra labai svarbūs, tačiau kraują siurbiančių vabzdžių kaip vektorių vaidmuo negali būti nuvertintas. Šie vabzdžiai daro įtaką visuomenės sveikatai, žemės ūkiui ir netgi pasaulinei prekybai, todėl jie yra labai svarbus entomologinių tyrimų objektas. Gilinant mūsų supratimą apie šiuos

vabzdžius, galime geriau pasiruošti ir kiek įmanoma mažinti rizikas, kurias jie kelia pasaulyje.

DARBO TIKSLAS IR UŽDAVINIAI

Darbo tikslas: Įvertinti kraujasiurbių vabzdžių – tikrųjų uodų (Culicidae) ir smulkiųjų mašalų (Ceratopogonidae, *Culicoides*) rūšių vaidmenį pernešant paukščių kraujo parazitus (Haemosporida (Apicomplexa), Trypanosomatida (Euglenozoa)).

Tikslui pasiekti buvo išsikelti šie uždaviniai:

1. Nustatyti Culicidae lervų įvairovę, sezoniškumą bei jų pasiskirstymo įvairiuose vandens telkiniuose priklausomybę nuo tam tikrų fizikinių bei cheminių parametrų.
2. Nustatyti tikrųjų uodų natūralaus užsikrėtimo paukščių kraujo parazitais (Haemosporida) ekstensyvumą taikant integratyvius tyrimo metodus.
3. Palyginti hibernuojančių ir aktyvių *Culex pipiens* tikrųjų uodų užsikrėtimo hemosporidiniais parazitais ir tripanosomomis ekstensyvumą.
4. Nustatyti smulkiųjų mašalų natūralaus užsikrėtimo paukščių kraujo parazitais (Haemosporida) ekstensyvumą taikant integratyvius tyrimo metodus.

GINAMIEJI TEIGINIAI

1. Tam tikrų tikrųjų uodų rūšių buvimas ir gausumas priklauso nuo vandens telkinio laikinumo, dugno padengimo, pH lygio, NO_3^- koncentracijos ir sezoniškumo.
2. *Coquillettidia*, *Culex* ir *Culiseta* genčių tikruosiuose uoduose dažniausiai aptinkama hemosporidinių parazitų DNR. *Ochlerotatus* genties rūšių tikruosiuose uoduose hemosporidinių parazitų ekstensyvumas yra mažiausias.
3. *Culex pipiens* yra galimas natūralus *Plasmodium matutinum* linijos pLINN1 vektorius, nes šis parazitas geba užbaigti sporogoninį vystymąsi šių tikrųjų uodų seilių liaukose.
4. Nustatyta, kad žiemojančiuose *Culex pipiens* uoduose nėra hemosporidinių parazitų ir tripanosomų (tik monokseninių tripanosomatidų). Aktyviuose *C. pipiens* uoduose aptinkama

paukščių (*Trypanosoma culicavium*), žinduolių (*T. trinaperronei*, *T. theileri*) tripanosomų ir paukščių hemosporidinių parazitų.

5. *Culicoides pictipennis*, *C. segnis* ir *C. kibunensis* smulkiuosiuose mašaluose aptiktų hemosporidinių parazitų ekstensyvumas – didžiausias.
6. *Culicoides segnis* yra galimas natūralus *Haemoproteus fringillae* hCCF3, *H. majoris* hPHSIB1, *H. asymmetricus* hTUPHI01 ir *H. minutus* hTURDUS2 vektorius. *Culicoides kibunensis* yra galimas natūralus *H. belopolskyi* hHIICT1, *H. homominutus* hCUKI1, *H. parabelopolskyi* hSYAT01 ir hSYAT02, ir *Haemoproteus* sp. hSYAT13 vektorius. *Culicoides reconditus* galimai yra natūralus *H. magnus* hROFI1 vektorius. *Culicoides pictipennis* yra *H. parabelopolskyi* hSYAT01, *Haemoproteus* sp. hSYAT13, *H. homogeneae* hSYAT16, *H. asymmetricus* hTUPHI01, ir *H. minutus* hTURDUS2 galimas natūralus vektorius, o *C. festivipennis* yra *H. belopolskyi* hHIICT1 galimas natūralus vektorius.

DARBO NAUJUMAS

1. Įrodyta, kad *Culex pipiens* tikrieji uodai yra galimi natūralūs *Plasmodium matutinum* genetinės linijos pLINN1 vektoriai, kadangi šios rūšies uodų seilių liaukose pirmą kartą mikroskopuojant aptikti *P. matutinum* pLINN1 sporozoitai.
2. *Plasmodium ashfordii* (pGRW02) pirmą kartą molekuliniu būdu aptikta kraujasiurbiuose vabzdžiuose (*Ochlerotatus sticticus*). Anksčiau ši genetinė linija buvo aptikta tik paukščiuose. Tai taip pat parodo, kad ši tikrųjų uodų rūšis minta paukščių krauju.
3. *Culex pipiens* tikruosiuose uoduose pirmą kartą aptikta *Trypanosoma trinaperronei*.
4. Pirmą kartą penkių hemosporidinių parazitų genetinių linijų sporozoitai buvo rasti *C. kibunensis*, penkių genetinių linijų *C. pictipennis*, keturių genetinių linijų *C. segnis* ir vienos genetinės linijos *C. festivipennis* seilių liaukose.
5. Pirmą kartą hemosporidinių parazitų genetinių linijų hCUKI1, hCULPIC02, hROFI1, hSYAT01, hSYAT13, hSYAT16 sporozoitai buvo rasti smulkiųjų mašalų seilių liaukose.
6. Pirmą kartą *C. reconditus* seilių liaukose aptikti *H. magnus* (hROFI1) sporozoitai.
7. Hibernuojančiuose *Culex pipiens* tikruosiuose uoduose nebuvo rasta hemosporidinių parazitų DNR. Dėl to tokie uodai gali būti

rekomenduojami eksperimentiniams paukščių maliarijos tyrimams naudojant gamtoje surinktų (ne laboratorijoje veisiamų) vabzdžių populiacijas.

LITERATŪROS APŽVALGOS APBENDRINIMAS

Literatūros apžvalgoje aptariami tikrųjų uodų bei smulkiųjų mašalų tyrimai, jų sistematika, biologija bei morfologija. Taipogi pateikiama informacija apie hemosporidinius parazitus, jį vystymąsi, daromą įtaką įvairiems organizmams bei kaip tikrieji uodai ir smulkieji mašalai dalyvauja jų pernešime. Taip pat pateikiama informacija apie tripanosomatidus, jų sukeliamas ligas, bei mokslinius tyrimus.

MEDŽIAGA IR METODAI

Tyrimų vietovės

Tikrųjų uodų lervos buvo surinktos 134-iuose Lietuvos vandens telkiniuose Vilniaus ir Alytaus miestų, Elektrėnų, Prienų, Šilutės ir Šiaulių rajonuose. Kiekvienos vietos koordinatės yra išsamiai pateiktos 1-oje papildomoje lentelėje. Tarp tirtų vandens telkinių buvo natūraliai pašlapusių vietų (ypatingai po pavasario atlydžio ar stipraus lietaus), įvairių balų, tvenkinių, vandens apsemtų griovių ar transporto priemonių vėžėse susidariusių balų.

Tikrųjų uodų suaugėliai buvo renkami Vilniaus mieste: Dvarčionyse (54°44'11.7"N 25°23'03.4"E), Belmonte (54°41'19.6"N 25°21'44.0"E), VU Kairėnų botanikos sode (54°44'02.2"N 25°24'13.2"E) ir Verkiuose (54°44'54.3"N 25°17'20.9"E); taip pat tikrieji uodai rinkti ir dviejose kaimo vietovėse: Puvočiuose (54°06'45.9"N 24°18'08.3"E) ir Brinkiškėse (54°47'55.9"N 25°03'35.7"E) (2.1.1. pav.).

Žiemojantys *Culex pipiens* buvo renkami drėgnuose rūsiuose Norkūnų (54°30'02.5"N, 23°56'24.2"E) ir Panemuninkėlių kaimuose (54°25'53.0"N, 24°04'05.7"E), taip pat rūsiuose Palangoje (55°55'05.8"N, 21°03'20.3"E) bei Vilniuje (bunkeriuose (54°42'01.6"N, 25°19'57.3"E) ir Verkių rūmų vandens bokšto rūsyje (54°44'56.9"N, 25°17'29.2"E)) (2.1.1. pav.).

Smulkiųjų mašalų suaugėliai buvo renkami įvairiose Lietuvos vietose: Verkių regioniniame parke (54°45′00″ N, 25°17′00″ E), VU Kairėnų botanikos sode (54°44′12.5″ N, 25°24′16.4″ E), Puvočiuose (54°06′52.2″ N,

24°18′17.6″ E), Ventės Rage (55°20′28.1″ N, 21°11′25.3″ E) ir Ventės Rago apylinkėse (55°23′57.5″ N, 21°14′14.8″ E ir 55°26′12.0″ N, 21°16′04.6″ E) bei Brinkiškėse (54°47′55″ N, 25°03′45″ E) (2.1.1. pav.).

Medžiagos rinkimas

Tikrųjų uodų (Culicidae) lervos

Tikrųjų uodų lervos buvo renkamos kovo–spalio mėnesiais (2021 metais) ir kovo–liepos mėnesiais (2022 metais). Mėginiai imti standartiniu samteliu (⌀12,5 cm) vieną–du kartus per mėnesį. Kiekvienas ėminys susidėjo iš trijų pakartojimų. Tikrųjų uodų lervos lauko tyrimų metu buvo sudedamos į atskirus indelius su 96% etanoliu.

Renkant tyrimo medžiagą buvo matuojama vandens temperatūra (°C), pH lygis bei nitritų (NO_2^- (mg/l)) ir nitratų (NO_3^- (mg/l)) koncentracijos. pH matavimams buvo naudojamos pH indikatorinės juostelės (Carl Roth, Vokietija), o NO_2^- ir NO_3^- koncentracijos buvo nustatytos naudojant nitratų ir nitritų indikatorių juosteles (Macherey-Nagel GmbH & Co, Vokietija). Kiekvienas vandens telkinys buvo suskirstytas į vieną iš trijų dydžio kategorijų: mažas (mažiau nei 4 m²), vidutinis (4–100 m²) ir didelis (daugiau nei 100 m²). Vandens telkinių dydis buvo matuojamas pavasarį.

Kiekvieno vandens telkinio dugno padengimas buvo įvertintas ir aprašytas kaip padengtas smėliu, žole, organinėmis medžiagomis (šakomis, lapais ir kt.) arba moliu. Be to, vandens telkiniai buvo apibūdinti ir pagal tai, ar jie yra atvirose vietose, kuriose juos pasiekia tiesioginiai saulės spinduliai, ar uždarose vietose, šešėlyje. Taip pat buvo užfiksuota, ar vandens telkiniai pastovūs ar laikini, t.y. ar jie išdžiūvo sezono metu, ar ne.

Tikrųjų uodų suaugėliai

Suaugę tikrieji uodai buvo renkami 2021–2023 metais (gegužės–lapkričio mėnesiais). 2021 metais tikrųjų uodų gaudymui buvo naudojamas entomologinis tinklelis. Mėginiai buvo renkami kas dvi savaites ir buvo atliekami trys pakartojimai po penkias minutes. Surinkti tikrieji uodai buvo sudėti į indelius su 96% etanoliu tolesniam transportavimui ir saugojimui iki identifikavimo bei tolimesnių tyrimų.

2022 ir 2023 metais buvo naudojamos CDC gaudyklės, su sausu ledu (CO_2 šaltinis). Gaudyklės kabintos likus 6–7 valandoms iki saulėlydžio ir

nuimtos 4–5 valandos po saulėtekio, o medžiaga iškart vežama į laboratoriją analizei.

Žiemojantys *Culex pipiens* uodai buvo renkami 2023–2024 metais gruodžio – sausio mėnesiais. Tikriesiems uodams aptikti buvo naudojami žibintuvėliai, o surinkimui – ekshausteriai. Medžiaga tą pačią dieną buvo vežama į laboratoriją tolesniam apdorojimui.

Culicoides smulkiųjų mašalų suaugėliai

Suaugę smulkieji mašalai buvo renkami 2021–2022 metais (birželio–rugsėjo mėnesiais). Tam tikslui buvo naudojamos UV LED šviesinės gaudyklės (BG-Pro All-In-One Biogents AG). Gaudyklės kabintos 1,5–2 m virš žemės tuo pačiu paros metu kaip ir tikrųjų uodų gaudyklės. Smulkieji mašalai buvo surinkti į mažus indelius su vandeniu ir lašeliu skysto muilo. Surinkta medžiaga buvo vežama į laboratoriją tolesnei analizei.

Vabzdžių rūšių identifikavimas ir skrodimas

Tikrųjų uodų lervų rūšių apibūdinimui buvo naudojamos 4-os stadijos lervos (kadangi šios stadijos metu visi rūšies identifikacijai reikalingi požymiai yra pilnai išsivystę), o identifikacijai naudotas Becker ir kt. (2003) morfologinio apibūdinimo raktas bei Gunay ir kt. (2018) interaktyvus tikrųjų uodų identifikavimo raktas „MosKeyTool“. Lervos buvo tiriamos stereomikroskopu (MOTIC SMZ-171, Motic, Kinija).

Pradžioje suaugę tikrieji uodai patalpinami 10–15 minučių į šaldiklį, kad taptų neaktyvūs. Išrūšiavus medžiagą ir atskyrus tikrųjų uodų pateles naudojant stereomikroskopą (MOTIC SMZ-171, Motic, China) tikrųjų uodų patelės apibūdinamos iki rūšies naudojantis tais pačiais raktais kaip ir apibūdinant tikrųjų uodų lervas. 2021 metų suaugusių uodų patelių pilveliai buvo sudėti po vieną arba kelis (2–5 tikrųjų uodų) į mėgintuvėlius tolimesniems molekuliniams tyrimams. 2022 ir 2023 kiekviena surinkta tikrųjų uodų patelė buvo tiriama atskirai. Nustačius tikrojo uodo patelės rūšį ji skrodžiama ir pagaminami du preparatai – seilių liaukų bei vidurinės žarnos, kurie išdžiovinami, užfiksuojami grynu metanoliu ir valandą dažomi su 4% Giemsa dažais (Valkiūnas, 2005; Žiegytė ir kt., 2017). Po skrodimų likusios vabzdžių dalys dedamos į atskirus mėgintuvėlius su SET buferiu tolimesniems molekuliniams tyrimams.

Surinkti hibernuojantys tikrieji uodai buvo patalpinami į šaldiklį, kad taptų neaktyvūs, *Culex pipiens* patelės atskiriamos nuo kitų rūšių patelių, taip

pat pagaminami seilių liaukų ir vidurinės žarnos preparatai, o vabzdžių likučiai (po 10 individų) molekuliniams tyrimams sudedami į mėgintuvėlius su SET buferiu.

Smulkiųjų mašalų suaugėliai atskirti pagal lytį, o tolimesniems tyrimams atrinktos tik tos patelės, kurios savo pilvelyje turėjo kraujo arba burgundiško pigmento, kuris leidžia atskirti bent kartą kiaušinėlius subrandinusias t.y. galimai krauju pasimaitinusias pateles. Pagaminti seilių liaukų preparatai bei pastovieji galvos ir sparnų preparatai, kadangi šios vabzdžio dalys naudotos rūšių identifikavimui. *Culicoides* rūšys apibūdintos naudojantis morfologiniais raktais (Glukhova ir kt., 1989; Gutsevich, 1973; Mathieu ir kt., 2012).

Mikroskopinių preparatų analizė

PGR teigiamų mėginių mikroskopavimas atliktas naudojant Olympus BX-43 šviesinį mikroskopą su Olympus DP12 skaitmenine kamera naudojant Olympus DP-SOFT (Olympus, Japan) programinę įrangą. Tikrųjų uodų ir smulkiųjų mašalų seilių liaukų preparatai buvo mikroskopuojami jeigu juose buvo aptikta hemosporidinių parazitų DNR, o tikrųjų uodų mėginiuose aptikus tripanosomų buvo mikroskopuoti tiek seilių liaukų tiek vidurinės žarnos preparatai. Teigiami preparatai bei smulkiųjų mašalų morfologiniai preparatai saugomi gamtos tyrimų centre, Vilniuje (sporozoitai smulkiųjų mašalų seilių liaukose: 49409NS–49425NS; NS49742–NS49757; sporozoitai tikrųjų uodų seilių liaukose: 49758NS; tripanosomos tikrųjų uodų preparatuose: 49805NS–49807NS).

Molekulinė analizė

DNR išskyrimas

Tikrųjų uodų, surinktų 2021 metais, žiemojančių *Culex pipiens* patelių ir smulkiųjų mašalų patelių DNR išskirta naudojant amonio acetato protokolą (Richardson ir kt., 2001). Likusių tikrųjų uodų DNR išskirta naudojant GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Lithuania) rinkinį, taikant gamintojo instrukcijas.

Polimerazės grandininė reakcija

Hemosporidinių parazitų paieška atlikta naudojant lizdinę PGR (Bensch ir kt., 2000; Hellgren ir kt., 2004). Tam tikslui naudoti HaemNFI ir HaemNR3 pradmenys pirmai PGR, o HaemF ir HaemR pradmenų pora panaudota antrai PGR. Šie pradmenys amplifikuoja genčių *Plasmodium* ir *Haemoproteus* mitochondrinės DNR citohromo *b* geno fragmentą.

Kai kurių tikruosiuose uoduose aptiktų hemosporidinių parazitų DNR sekų chromatogramose buvo matomi daugybiniai pikai, kurie parodo galimas mišrias infekcijas. Tokiais atvejais buvo panaudotas Ciloglu ir kt. (2019) sukurtas PGR protokolas. Šiuo protokolu amplifikuojami skirtingų ilgių mitochondrinės DNR fragmentai: *Plasmodium* – 377–379 bp (pradmenų pora: PMF ir PMR), *Haemoproteus* – 525–533 bp (pradmenų pora: HMF ir HMR) ir *Leucocytocoon* – 218 bp (pradmenų pora: LMF ir LMR). Atlikus PGR produkto elektroforezę 2% agarozės gelyje buvo nustatyta kurie mėginiai turėjo kelias skirtingų ilgių juostas, reiškiančias mišrią infekciją skirtingų genčių parazitais.

Ieškant tripanosomų *Culex pipiens* uoduose taip pat buvo naudojamas lizdinės PGR protokolas (Sehgal ir kt., 2015; Valkiūnas ir kt., 2011), amplifikuojantis 18S rRNR koduojantį DNR fragmentą. Tam buvo naudotos dvi pradmenų poros (pirmoji: Tryp763 ir Tryp1016, antroji: Tryp99 ir Tryp957).

Kai kurių *Culicoides* smulkiųjų mašalų rūšių patvirtinimui buvo naudojamas Folmer ir kt. (1994) parengtas PGR protokolas amplifikuojantis citochromo *c* oksidazės pirmojo subvieneto (COI) mitochondrinės DNR fragmentą. Tam naudota LCO1490 ir HCO2198 pradmenų pora.

Kadangi tikrųjų uodų rūšių *Culex pipiens* ir *Culex torrentium* patelių morfologiškai neįmanoma atskirti, tam tikslui buvo naudojamas Hesson ir kt. (2010) parengtas protokolas, amplifikuojantis mitochondrinės DNR COI fragmentą. Pradžioje atliekama PGR naudojant C1-J-2183 ir TL2-N-3014 pradmenų porą (Simon ir kt., 1994). Gautas PGR produktas inkubuojamas, kartu su SspI arba FspBI restriktazėmis, 5-ioms minutėms 37°C temperatūroje (reagentų kiekiai taikyti naudojant gamintojo ThermoFisher Scientific instrukcijas). Restriktazė SspI *C. pipiens* fragmentą perkerpa į du fragmentus (~620 pb ir 210 bp), o *C. torrentium* lieka neperkirptas, o naudojant FspBI restriktazę – atvirkščiai. Šis metodas leidžia atskirti rūšis iš elektroforezės rezultatų, tuomet nereikia atlikti sekoskaitos ir analizuoti gautų sekų.

Elektroforezė

PGR produktai vizualizuoti 2% agarozės gelyje, naudojant MidoriGreen dažus (NIPPON Genetics Europe, Germany). Teigiamų mėginių (hemosporidinių parazitų atveju turintys ~500 bp ilgio juostelę, tripanosomų ~750 bp ir ~600 bp vabzdžių) produktai buvo išgryninti ir parengti sekoskaitai naudojant amonio acetato protokolą (Sambrook and Green, 2012). Sekoskaita atlikta naudojant įrangą Applied Biosystems Genetic Analyzer 3500.

Sekų analizė

Naudojant programinę įrangą Geneious Prime 2023.2.1 (https://www.geneious.com) ir BioEdit 7.7.1. gautos sekos buvo analizuojamos bei įvertinta jų kokybė. Naudojant BLAST funkciją MalAvi duomenų bazėje (http://mbio-serv2.mbioekol.lu.se/Malavi) mūsų gautos hemosporidinių parazitų sekos buvo lyginamos ir ieškoma atitinkančių parazitinių organizmų sekų, taip pat gauti rezultatai patikrinti naudojant BLAST funkciją NCBI duomenų bazėje (http://www.ncbi.nlm.nih.gov/BLAST). Hemosporidinių parazitų atveju 100% atitinkančios sekos buvo laikomos teigiama egzistuojančių rūšių ir genetinių linijų identifikacija, kai tuo tarpu sekos, kurios skiriasi bent viena bazių pora ir neturi atitikmenų ankstesniuose tyrimuose bei minėtose duomenų bazėse buvo laikomos naujomis genetinėmis linijomis (Bensch ir kt., 2009). Gautos sekos buvo deponuotos genų banke (sekų numeriai pateikti lentelėje 2.7.1.).

Tripanosomatidų filogenetinis medis buvo nubraižytas naudojant Geneious Prime 2023.0.4 programinę įrangą. Geriausias filogenetinės analizės modelis (GTR+I+G) buvo nustatytas naudojant jModeltest-2.1.10 programinę įrangą. Tam taip pat naudotas MrBayes įskiepis v3.2.6.

Statistinė analizė

Vidutinis tikrųjų uodų lervų skaičius ėminyje, standartinės paklaidos (SE) buvo apskaičiuotos naudojant Microsoft Excel 365, 12-ai dažniausiai aptiktų tikrųjų uodų rūšių taip pat paskaičiuota daugybinė regresija naudojant programinę įrangą STATISTICA 12.5. Naudojant Brodgar 2.7.5. programinę įrangą (Highland Statistics Ltd.) buvo atlikta daugiamatė perteklinė analizė (RDA) parodanti tikrųjų uodų rūšių ir matuotų parametrų ryšius.

Suaugusiuose tikruosiuose uoduose aptiktų sporozoitų pločio, ilgio ir ploto vidutinis ir standartiniai nuokrypiai (SD) buvo apskaičiuoti naudojant Microsoft Excel 365. Norint įvertinti bendrą tikrųjų uodų užsikrėtimą, buvo

apskaičiuotas minimalus užsikrėtimo dažnis (MIR) (Schoener ir kt., 2017). Analizuojant sutelktinius tikrųjų uodų mėginius, jei mėginyje buvo aptikta hemosporidinių parazitų DNR, buvo daroma prielaida, kad vienas tikrasis uodas iš mėginio buvo užsikrėtęs:

$$MIR\ (\%) = n_{(teigiami\ mėginiai)} / N_{(visi\ tirti\ tikrieji\ uodai)} \times 100$$

Chi kvadratas (nepriklausomumo testas) buvo apskaičiuotas siekiant palyginti hemosporidinių parazitų ekstensyvumą įvairiose tikrųjų uodų rūšyse. Žiemojančių ir aktyvių *Culex pipiens* uodų palyginimas atliktas taikant Fischerio testą. Abiejų testų *P* vertė < 0,05 buvo laikoma reikšminga.

Siekiant suprasti, kaip Europoje sąveikauja įvairių rūšių smulkieji mašalai ir juose aptinkamos *Haemoproteus* spp. genetinės linijos, buvo naudojami iki šiol Europoje paskelbti tyrimai. Šių tyrimų publikacijų ieškota naudojant PubMed ir Google Scholar duomenų bazes su paieškos terminais: „Culicoides AND Haemosporida“, „Culicoides AND Haemosporidian“, „Culicoides AND Haemoproteus“ ir „Haemoproteus AND vectors“. Į šią analizę buvo įtraukti tik tie tyrimai, kuriuose buvo naudojami molekuliniai tyrimų metodai ir amplifikuotas *cytb* geno fragmentas. Buvo padaryta *Culicoides* rūšių ir *Haemoproteus* genetinių linijų sąveikų matrica. Sąveikų tinklas ir matrica buvo sukonstruoti naudojant R 4.0.5 „Bipartite“ paketą.

Chi kvadratas (nepriklausomumo testas) buvo apskaičiuotas siekiant palyginti hemosporidinių parazitų ekstensyvumą įvairiose smulkiųjų mašalų rūšyse.

REZULTATAI IR JŲ APTARIMAS

Tikrųjų uodų lervų rūšių įvairovė, sezoniškumas ir ryšiai tarp lervų gausumo ir fizikinių bei cheminių vandens telkinių parametrų

Tyrimo metu iš viso surinkti 606 mėginiai, iš kurių 225-iuose aptiktos tikrųjų uodų lervos. Mėginiai rinkti 134-iuose vandens telkiniuose, iš kurių 101-ame vandens telkinyje bent vieną kartą aptiktos tikrųjų uodų lervos. Pirmą kartą tikrųjų uodų lervos aptiktos kovo 31 dieną ir buvo aptinkamos iki rugsėjo 30 dienos. Iš viso surinktos 5 392 lervos, o 2 141 lerva buvo ketvirto ūgio ir buvo apibūdinta iki rūšies. Nustatytos 25 tikrųjų uodų rūšys, priklausančios penkioms gentims (lentelė 3.1.1.).

Tyrimo metu gausiausiai buvo aptinkami *Ochlerotatus* spp. (67,6%) ir *Aedes* spp. (25,2%) tikrieji uodai. Viename mėginyje vidutiniškai buvo aptinkamos 8,9±1,5 tikrųjų uodų lervos. Daugiausiai (50,6%) tikrųjų uodų lervų buvo surinkta balandžio mėnesį (3.1.1.1. pav.), tačiau skirtingos rūšys buvo randamos nuo kovo pabaigos iki rugsėjo pabaigos. *Aedes* uodai turėjo du sezoninio gausumo pikus, o *Culiseta* ir *Culex* rūšys buvo dažniausiai aptinkamos antroje vasaros pusėje. *Ochlerotatus* rūšių uodų lervos dažniausiai buvo randamos pavasarį ir vasaros pradžioje, išskyrus *O. sticticus*, kuri turėjo dvi kartas, o šios rūšies tikrųjų uodų gausumo pikas buvo liepos mėnesį (3.1.1.4 lentelė).

Iš visų mėginių 64,3% atvejais buvo aptinkama tik viena tikrųjų uodų rūšis. *Anopheles maculipennis* ir *Ochlerotatus riparius* niekuomet nebuvo aptikta su kitomis rūšimis kartu (3.1.1.2 lentelė). Tik dviem atvejais tame pačiame mėginyje buvo aptiktos keturios rūšys.

Tyrimas parodė, kad sezoniškumas, vandens telkinių pastovumas, nitratų lygis, pH ir dugno padengimas yra susiję su tikrųjų uodų lervų buvimu ar nebuvimu bei jų gausumu, tačiau saulės šviesa ir vandens telkinių dydis nėra statistiškai reikšmingi. Kai kurių rūšių uodai, tokių kaip *Aedes vexans* ir *O. annulipes,* buvo randami esant plačiam temperatūrų ir pH diapazonui. Priešingai, *O. cantans, O. cataphylla* ir *Aedes cinereus* buvo randamos siauresniuose diapazonuose (3.1.1.2. pav.). Nitratų koncentracijos svyravo nuo 0 iki 50 mg/L, o nitritų nuo 0 iki 0,5 mg/L, tačiau didžiojoje dalyje (91%) vandens telkinių nitratai ir nitritai neaptikti. Dažniausiai pasitaikęs (31,3%) vandens telkinių dugno padengimas – mišri organika, daugiau nei pusė (52,3%) vandens telkinių buvo vidutinio dydžio, o 64,7% vandens telkinių buvo pavėsyje (3.1.1.3. pav.).

Daugybinės regresijos analizė parodė kai kurių rūšių gausumo ryšius su sezoniškumu: *Culex territans* lervos aptiktos tik nuo liepos pabaigos, o *O. punctor* tik balandžio mėnesį (lentelės 3.1.1.3., 3.1.1.4.). *Aedes cinereus* ir *Culiseta annulata* dažniau aptikta laikinuose vandens telkiniuose, o pastaroji rūšis rasta vandens telkiniuose, kurių dugnas padengtas organika (lentelė 3.1.1.3., 3.1.1.3. pav.). Nitratų kiekis vandens telkinyje turėjo neigiamą ryšį su *Culex pipiens* gausumu ir teigiamą su *O. cantans* lervų gausumu (lentelė 3.1.1.3.). Šiuos daugybinės regresijos analizės duomenis taip pat pagrindė daugiamatės perteklinės analizės rezultatai (3.1.1.4. pav.).

Diskusija

Šiame tyrime nustatytos 25 tikrųjų uodų rūšys, o tai sudaro 69,4% visų Lietuvoje aptinkamų Culicidae šeimai prisikiriamų rūšių (Bernotienė ir Lučiūnaitė, 2011; Pakalniškis ir kt., 2006). Ankstesniuose Podėnaitės (1959; 1962) tyrimuose buvo atitinkamai aptiktos 26 ir 22 tikrųjų uodų rūšys (lentelė 3.1.2.1.), o tai rodo, kad tolesnių tyrimų ir stebėjimų įvairiose buveinėse metu galima aptikti daugiau tikrųjų uodų rūšių.

Ochlerotatus cataphylla ir *O. cantans* dažniausiai buvo aptinkamos kartu (15,9 % rūšių porų). Becker ir kt. (2003) pažymėjo, kad *O. cataphylla* gausiau vystosi neutraliame ir silpnai šarminiame vandenyje, tačiau mūsų duomenys parodė, kad šios rūšies lervos buvo rastos silpnai rūgštingame (pH = 6) vandenyje, o *O. cantans* lervos buvo aptinkamos vandens telkiniuose, kurių pH vertės vidutiniškai siekė 6,9. *Ochlerotatus cataphylla* ir *O. cantans* taip pat siejami su žema vandens temperatūra, kuri leidžia jiems vystytis anksti pavasarį, kuomet vanduo Šiaurės Europos šalių vandens telkiniuose yra šaltas (Becker ir kt., 2003; Wegner, 2009b), o tai sutampa su mūsų gautais rezultatais – daugiausia tikrųjų uodų lervų aptikta balandį. Ir atvirkščiai, aukštesnei vandens temperatūrai pirmenybę teikė rūšys, priklausančios *Culex* ir *Culiseta* gentims. Šios rūšys vystosi vasarą, kai vandens temperatūra yra aukštesnė (išskyrus *Culiseta morsitans*). Tai, kad *Culiseta* genties uodai dažniau vystosi aukštoje vandens temperatūroje nurodo ir kiti autoriai. *Culiseta morsitans* lervos gali vystytis įvairiuose vandens telkiniuose; šios rūšies tikrieji uodai žiemoja lervos stadijoje ir virsta lėliukėmis tik po pavasario (Becker ir kt., 2003). Oliver ir Howard (2011) ištyrė, kad šiaurėje *C. morsitans* žiemoja kiaušinėlių stadijoje, o šios rūšies lervoms vystytis reikalinga aukšta vandens temperatūra. Mūsų tyrime šios rūšies lervos buvo aptinkamos balandžio mėnesį (daugiau nei trečdalis visų rastų lervų), o vėliau vėl pasirodė nuo birželio iki rugsėjo.

Šiame tyrime *Ochlerotatus punctor* lervos buvo aptiktos balandžio mėnesį, kuomet vidutinė vandens temperatūra dar būna ganėtinai žema, kai tuo tarpu *Culex territans* lervos buvo aptinkamos antroje vasaros pusėje, kuomet vandens temperatūra yra aukšta. Becker ir kt. (2003) teigia, jog *O. punctor* priklauso ekologinei „sniego tirpsmo“ tikrųjų uodų grupei, šios lervos pradeda vystytis anksti pavasarį, o *C. territans* galima aptikti nuo ankstyvo pavasario iki rugsėjo, o gausumo pikas – vasaros pabaigoje. *Culex pipiens, C. territans, Culiseta annulata, O. flavescens* ir *O. sticticus* tikrųjų uodų rūšys mūsų tyrime buvo aptiktos tik vandens telkiniuose, kuriuose nitratų ir nitritų neaptikta. Laboratorinių tyrimų metu buvo pastebėta, jog *C. pipiens* kiaušinėlių dėjimui dažniau renkasi vandens indelius su mažesniu nitratų kiekiu (Dowling ir kt., 2013).

Pasak Becker ir kt. (2003) ir Wegner (2009b), kai kurios tikrųjų uodų rūšys kiaušinėlius deda vieną kartą per sezoną, pavyzdžiui, ankstyvo pavasario rūšys (pvz. *O. cataphylla*). Tai atitinka mūsų rezultatus, nes dauguma *O. cataphylla* lervų buvo rastos balandį, kai kurios – gegužę. Panašiai ir *O. annulipes* yra žinoma kaip rūšis, dedanti kiaušinėlius vieną kartą per sezoną (Becker ir kt., 2003), o tai sutampa su mūsų tyrimų rezultatais, nes ši rūšis buvo aptikta balandžio–gegužės mėnesiais. Yra žinoma, kad *O. cantans* turi vieną kartą per sezoną šiaurinėje Europos dalyje, tačiau piečiau esančiose šalyse gali turėti dvi kartas (Becker ir kt., 2003; Wegner 2009b). Mūsų rezultatai parodė dvi aiškias *O. cantans* kartas: pirmoji – balandžio–gegužės, o antroji – rugpjūčio–rugsėjo mėnesiais (3.1.1.4. lentelė). Šiaurinėje Europos dalyje *Culex territans* turi tik vieną kartą per sezoną (Becker ir kt., 2003), tačiau panašu, jog Lietuvoje yra gana ilgas šios rūšies vystymuisi palankus sezonas su keliomis *C. territans* kartomis, nes šios rūšies lervos buvo aptinkamos nuo birželio iki rugpjūčio. Pasak Wegner (2009b), manoma, kad *O. sticticus* turi vieną kartą sezono metu, o Becker ir kt. (2003) teigė, kad tai kelias kartas per sezoną turinti rūšis. Šią rūšį aptikome balandžio–gegužės ir liepos mėnesiais, o birželį lervų nerasta, o tai parodo, kad per sezoną išsivystė mažiausiai dvi kartos. Wegner (2009b) straipsnyje rašo, kad *O. punctor* turi kelias kartas per metus, tačiau šio tyrimo metu šios rūšies lervos buvo rastos tik balandžio mėnesį.

Aedes cinereus – holarktiniame regione paplitusi rūšis, kuri Lietuvos sąlygomis gali turėti kelias kartas (3.1.1.4. lentelė). Šiaurinėje Europos dalyje ši rūšis turi vieną kartą, bet piečiau gali turėti kelias kartas per metus (Wegner, 2009b). Šios rūšies lervos išsirita kiek vėliau, nes jų lervų vystymuisi reikalinga aukštesnė temperatūra nei tipinėms „sniego tirpsmo“ rūšims (Becker ir kt., 2003). Tai gali paaiškinti, kodėl šios rūšies lervos buvo rastos balandį, bet didžiausią gausumą pasiekė tik gegužę, kuomet vidutinė vandens temperatūra siekė 12,0 °C, kai tuo tarpu *O. cantans* uodų lervos buvo aptikamos 8,7°C vidutinėje temperatūroje ir *O. punctor* – 8,2 °C. *Aedes vexans* yra kelias kartas per sezoną turinti rūšis, laikoma „vasarine“ rūšimi, nes žinoma, kad jos optimali vystymosi temperatūra yra apie 30 °C (Becker ir kt., 2003; Wegner, 2009b). Tai sutampa su mūsų tyrimo rezultatais, nes nedaug šios rūšies tikrųjų uodų lervų aptikta balandžio–gegužės mėnesiais, o pikas stebėtas liepos mėnesį, kuomet vidutinė vandens telkinių temperatūra buvo aukščiausia (3.1.1.4 lentelė).

Culex pipiens yra laikoma viena iš labiausiai visame holarkties regione paplitusių tikrųjų uodų rūšių (Wegner, 2009b; Weitzel ir kt., 2011), tačiau mūsų tyrime šios rūšies lervos buvo aptiktos retai ir sudarė tik 2,3% visų

surinktų ir nustatytų tikrųjų uodų lervų rūšių. Gali būti, kad palankiausios *C. pipiens* lervų buveinės šio tyrimo metu nebuvo tirtos, kadangi kiti tyrimai rodo, jog suaugę *C. pipiens* uodai yra gana gausiai aptinkami Lietuvoje (Valavičiūtė-Pocienė ir kt., 2024).

Kadangi dauguma tyrimų daugiausia dėmesio skiria suaugusioms tikrųjų uodų patelėms dėl jų maitinimosi, elgsenos ir įvairių pernešamų patogenų, manome, kad taip pat svarbu tirti lervų stadijas. Tai padeda geriau suprasti tikrųjų uodų rūšių įvairovę ir gali suteikti naujų įžvalgų tikrųjų uodų gausumo reguliavimo strategijose (Gunathilaka ir kt., 2019; Vantaux ir kt., 2016; Westbrook ir kt., 2010).

Natūralus tikrųjų uodų užsikrėtimas paukščių kraujo parazitais (Haemosporidae)

2021–2023 metais buvo ištirtos 2 820 tikrųjų uodų patelės. Buvo aptiktos 14 rūšių, priklausančių penkioms gentims (3.2.1.1. pav.). Šio tyrimo metu penkių tikrųjų uodų rūšių patelėse (*Aedes cinereus, Coquillettidia richiardii, Culex pipiens, Ochlerotatus cantans* ir *O. sticticus*) buvo nustatyti penkių rūšių ir genetinių linijų *Plasmodium* parazitai bei trijų rūšių ir genetinių linijų *Haemoproteus* parazitai (lentelė 3.2.1.1.).

Vieno *Culex pipiens* uodo seilių liaukose pirmą kartą aptikti *Plasmodium matutinum* pLINN1 sporozoitai (3.2.1.2. pav.). Tai parodo, jog šios rūšies parazitai geba užbaigti sporogoninį vystymąsi šios rūšies tikrųjų uodų seilių liaukose. Šių sporozoitų ilgis varijavo tarp 8,1 ir 11,2 µm (vid. 9,6 µm, SD±0,7), plotis nuo 0,6 iki 1,0 µm (vid. 0,8 µm ±1,0), plotas nuo 4,3 iki 9,5 µm² (vid. 6,9 µm² ±1,2).

Bendras tikrųjų uodų užsikrėtimo ekstensyvumas siekė 2,0%, o liepos (3,6%) ir rugpjūčio (2,9%) mėnesiais buvo aukščiausias. Labiausiai hemosporidiniais sporozoitais užsikrėtusios rūšys – *Coquillettidia richiardii* (3,5%) ir *Culex pipiens* (2,9%) rūšys, bei gentis *Culiseta* (8,7%) (3.2.1.1. pav.).

Šiame tyrime net 30,7% teigiamų mėginių sekų turėjo daugybinius pikus, indikuodami, kad tai gali būti mišrios infekcijos. Mišrios infekcijos *Plasmodium, Haemoproteus* ir *Leucocytozoon* genčių parazitais buvo nustatytos aštuoniuose *C. richiardii* rūšies tikruosiuose uoduose, keturi tos pačios rūšies tikrieji uodai buvo užsikrėtę *Plasmodium* ir *Haemoproteus* genčių parazitais, o trys *C. richiardii* ir vienas *C. pipiens* rūšių tikrieji uodai buvo užsikrėtę *Plasmodium* ir *Leucocytozoon.*

Diskusija

Šiame tyrime gauti rezultatai parodo, kad vidutinio klimato juostoje *Aedes cinereus, Coquillettidia richiardii, Culex pipiens, Culiseta alaskaensis, Culiseta morsitans, Ochlerotatus cantans* ir *O. sticticus* galimai atlieka svarbų vaidmenį paukščių kraujo parazitų pernešime, kadangi nustatytos parazitų rūšys vystosi būtent paukščių kraujyje. Visos šiame tyrime aptiktos genetinės linijos skyrėsi nuo prieš tai nustatytų Lietuvoje (Bernotienė ir Valkiūnas, 2016).

Culex pipiens uodo seilių liaukose pirmą kartą aptikti *Plasmodium matutinum* pLINN1 sporozoitai. Prieš tai *C. pipiens* uoduose pLINN1 buvo aptikta tik taikant PGR metodus (Ferraguti ir kt., 2013a; Kimura ir kt., 2010; Martínez-de la Puente ir kt., 2015; Schoener ir kt., 2017; Zélé ir kt., 2014). *Culex pipiens* dėl savo ornitofilinio gyvenimo būdo yra svarbus paukščių maliarijos vektorius (Farajollahi ir kt., 2011; Martínez-de la Puente ir kt., 2015). *Coquillettidia* genties tikrieji uodai taip pat yra svarbūs paukščių kraujo parazitų pernešime, o tyrimai rodo didelį užsikrėtimo lygį (Njabo ir kt., 2009, 2011; Schoener ir kt., 2017).

Šiame tyrime bendras tikrųjų uodų užsikrėtimo ekstensyvumas siekė 2,0%, kai tuo tarpu tyrimuose Madagaskare jis siekė 5,02% (Schmid ir kt., 2017), Austrijoje 6,43% sutelktinių mėginių aptikta hemosporidinių parazitų DNR (Schoener ir kt., 2017). Turkijoje 10,7% sutelktinių mėginių buvo teigiami (Inci ir kt., 2012).

Plasmodium homonucleophilum (pSW2) šiame tyrime buvo dažniausiai aptinkama genetinė linija. Prieš tai ši genetinė linija buvo aptikta *C. pipiens* uoduose Šveicarijoje (Glaizot ir kt., 2012) bei įvairiose paukščių rūšyse Europoje ir Azijoje (Nourani ir kt., 2020). *Plasmodium matutinum* (pLINN1) DNR šiame tyrime buvo aptikta keturiuose tikruosiuose uoduose, tačiau ši genetinė linija yra plačiai paplitusi Europoje, Amerikoje, Naujojoje Zelandijoje bei Japonijoje ir yra aptinkama 16-ai šeimų priklausančiose paukščių rūšyse (http://130.235.244.92/MalAvi/; informacija gauta 2024 balandžio 24). *Plasmodium circumflexum* (pTURDUS1) šiame tyrime buvo rasta *O. sticticus* uoduose, o prieš tai ją aptiko *C. pipiens* uoduose Šveicarijoje (Glaizot ir kt., 2012). *Plasmodium ashfordii* (pGRW02) pirmą kartą buvo aptikta vabzdyje (*O. sticticus*), prieš tai ši genetinė linija buvo aptikta tik paukščiuose Europoje, Afrikoje, ir Azijoje (http://130.235.244.92/MalAvi/; informacija gauta 2024 balandžio 24). *Plasmodium vaughani* (pSYAT05) buvo aptikta *Coquillettidia richiardii* ir *Culex pipiens* uoduose, o pasaulyje ši

genetinė linija yra plačiai aptinkama tiek vabzdžiuose, tiek paukščiuose (http://130.235.244.92/MalAvi/; informacija gauta 2024 balandžio 24).

Haemoproteus parazitų aptikimas *C. pipiens* ir *O. sticticus* uoduose parodo, jog šie vabzdžiai maitinosi paukščių krauju, kadangi aptiktų parazitų rūšys vystosi paukščiuose. Tačiau iki šiol nėra įrodymų, kad uodai geba pernešti *Haemoproteus* parazitus.

Šis tyrimas pabrėžia mikroskopijos ir molekulinių tyrimų derinimo svarbą, kad būtų galima geriau suprasti tikrųjų uodų rūšių gebėjimą pernešti paukščių kraujo parazitus. Šių tyrimų rezultatai parodo, jog *Ochlerotatus* genties uodai atlieka nedidelį vaidmenį hemosporidinių parazitų pernešime, o gentims *Aedes, Culex, Culiseta* ir *Coquillettidia* turėtų būti skiriamas didesnis dėmesys planuojant tyrimus ateityje.

Culex pipiens uodų užsikrėtimas tripanosomomis bei paukščių kraujo parazitų paieška žiemojančiuose *C. pipiens* uoduose

Tyrimo metu buvo išanalizuoti 1 037 *C. pipiens* uodai: 556 surinkti šiltojo sezono metu (aktyvūs uodai) ir 481 žiemojantys (hibernuojantys) tikrieji uodai. Mūsų tyrime nustatytas 2,9% užsikrėtimo hemosporidiniais parazitais ekstensyvumas aktyviuose *C. pipiens* uoduose (lentelė 3.2.1.1.). Tuo tarpu hemosporidinių parazitų DNR hibernuojančiuose tikruosiuose uoduose neaptikta.

Šiltuoju sezono metu 1,6% *C. pipiens* uodų (devyni individai) buvo aptikta tripanosomų DNR (*Trypanosoma culicavium, T. theileri, T. trinaperronei*), o 0,4% (du individai) žiemojančiuose tikruosiuose uoduose aptikti monokseniniai tripanosomatidai (lentelė 3.3.1.1.).

Mikroskopuojant *C. pipiens* seilių liaukose buvo aptikta *T. culicavium* (3.3.1.2.A pav.), *T. trinaperronei* buvo aptikta tiek seilių liaukose, tiek vidurinėje žarnoje (3.3.1.2.B pav.), monokseniniai tripanosomatidai buvo rasti vidurinės žarnos preparate (3.3.1.2.C pav.), o *T. theileri* tikrųjų uodų žarnų ar seilių liaukų preparatuose nebuvo aptikta.

Diskusija

Šiame tyrime nustatytas hemosporidinių parazitų ekstensyvumas *Culex pipiens* uoduose lyginant su kitų Europos šalių tyrimais yra vidutinis. Kitų autorių šaltiniuose minimas užsikrėtimas svyruoja nuo 0,04% iki 6,6% (0,04% – (Ventim ir kt., 2012); 0,52% – (Ejiri ir kt., 2009); 0,61% – (Synek ir kt.,

2013); 3,08% – (Kim ir Tsuda, 2010); 6,6% – (Glaizot ir kt., 2012)). Tuo tarpu hemosporidiniais parazitais užsikrėtusių žiemojančių tikrųjų uodų neaptikta.

Žinios apie hemosporidinių parazitų aptikimą hibernuojančiuose tikruosiuose uoduose yra labai ribotos. *Plasmodium matutinum* (pLINN1) buvo aptikta sutelktiniuose žiemojančių tikrųjų uodų, surinktų lapkričio mėnesį, mėginiuose naudojant molekulinius tyrimų metodus (Köchling ir kt., 2023). Ši parazito linija dažnai aptinkama *C. pipiens* uoduose, surinktuose šiltuoju periodu, naudojant molekulinius tyrimo metodus (2-a papildoma lentelė). Mes sutinkame su Köchling ir kolegomis (2023), jog *Plasmodium* galimai geba peržiemoti tikruosiuose uoduose, tačiau norint tai įrodyti reikia papildomų eksperimentinių ir integratyvių tyrimų. Vidurinėje klimato juostoje lapkritis yra mėnuo, kuomet kai kurios tikrųjų uodų rūšys pradeda žiemojimą (Becker et al., 2003), o tam, kad būtų parazitų rezervuarais, tikrųjų uodų patelės turėtų išgyventi su parazitais iki šiltojo sezono pradžios (balandžio–gegužės mėn.) ir pasimaitinti tinkamo šeimininko krauju.

Culex pipiens uodai yra laikomi ornitofiliniais (Farajollahi ir kt., 2011; Martínez-de la Puente ir kt., 2015). Aptiktos *Plasmodium* rūšys ir genetinės linijos tirtose *C. pipiens* patelėse patvirtina faktą, jog jos maitinosi paukščių krauju. *Trypanosoma culicavium* taip pat parazituoja paukščius (Votypka ir kt., 2012), dar labiau pagrįsdama šios rūšies tikrųjų uodų ornitofilinę mitybą, tačiau *T. theileri* (Brotánková ir kt., 2022) ir *T. trinaperronei* (Garcia ir kt., 2020) vystosi stambių žinduolių kraujyje, taigi šių parazitų aptikimas *C. pipiens* uoduose parodo, kad ši rūšis mitybiniu požiūriu yra oportunistinė. Taip įvairių grupių gyvūnų krauju besimaitinantys tikrieji uodai gali pernešti parazitinius organizmus iš vienų gyvūnų rūšių kitoms gyvūnų rūšims ir tapti tiltu patogenų pernešime, kaip kad pvz. Vakarų Nilo virusas įprastai cirkuliuoja paukščių populiacijose, tačiau tikrieji uodai juos gali perduoti ir žmonėms, o tai dažnai sukelia ir sunkius susirgimus (Hamer ir kt., 2014). Yra nustatyti žmonėms netipiniai užsikrėtimai gyvūnų tripanosomomis, kuomet buvo sukelti sunkūs susirgimai ar netgi mirtys (Truc ir kt., 2013).

Culex pipiens uoduose aptikta tripanosomatidų įvairovė yra pateikiama 3-ioje pepildomoje lentelėje. Yra žinomos dvi rūšys – *T. culicavium* (Votypka ir kt., 2012; Zídková ir kt., 2012) ir *T. thomasbancrofti* (Fialová ir kt., 2021; Šlapeta ir kt., 2016; Zídková ir kt., 2012), kurias geba pernešti *C. pipiens* uodai (Fialová ir kt., 2021). Šios rūšies tikrųjų uodų patelėse buvo aptinkamos ir kitos tripanosomatidų rūšys: *T. avium* (Schoener ir kt., 2019; 2018; Svobodová ir kt., 2015), *T. theileri* (Brotánková ir kt., 2022) bei tokie monokseniniai tripanosomatidai kaip *Crithidia brevicula* (Svobodová ir kt., 2015), *C. dedva* (Schoener ir kt., 2019), *C. fasciculata* (Schoener ir kt., 2018),

C. dobrovolskii), *Strigomonas* cf. *oncopelti*, *Paratrypanosoma* cf. *confusum*, bei iki šiol neapibūdintas Trypanosomatidae atstovas (Kostygov ir kt., 2022). Šie rezultatai yra verti dėmesio, kadangi gamtoje pagrindiniai vektoriais yra laikomi Simuliidae upiniai mašalai (Votýpka ir kt., 2002), tačiau ir kelios *Culicoides* rūšys nustatytos kaip potencialūs vektoriai (Bernotienė ir kt., 2020; Kazak ir kt., 2023).

Trypanosoma theileri buvo viena pirmųjų žinduoliuose apibūdintų tripanosomų, kurių vieni iš pagrindinių pernešėjų – briedmusės (Garcia ir kt., 2020) ir sparvos (Böse ir kt., 1987). Nors duomenų apie šio parazito paplitimą tikruosiuose uoduose nėra daug, bet kai kuriuose naujesniuose tyrimuose *C. pipiens* uoduose buvo aptiktas nedidelis (0,05%) užsikrėtimas, tačiau *Aedes* uoduose jis siekė 22,0% (Brotánková et al., 2022). Mūsų rezultatai rodo kiek didesnį (0,72%) aptikimo dažnį.

Šiuo metu žinoma, jog kelios *Megatrypanum* tripanosomų rūšys parazituoja elnius, įskaitant *Trypanosoma mazamarum* (Mazza ir kt., 1932), *T. cervi* (Kingston ir Moston, 1975), *T. stefanskii* (Kingston ir kt., 1992) ir *T. trinaperronei* (Garcia ir kt., 2020). Kartu su *T. theileri*, T. *melophagium*, *T. cervi* ir *T. trinaperronei* sudaro kompleksą, žinomą kaip *Trypanosoma theileri* kompleksas (Garcia ir kt., 2011; 2020; Rodrigues ir kt., 2006). Briedmusės (*Lipoptena cervi* ir *L. mazamae*) yra laikomos *T. trinaperronei* pernešėjomis (Garcia ir kt., 2020), ir šie parazitai anksčiau niekada nebuvo rasti tikruosiuose uoduose. Tiek molekuliniais metodais, tiek mikroskopuojant mes aptikome viename *C. pipiens* uode *T. trinaperronei*. Gauta seka rodo 99,7% panašumą su seka, deponuota GenBank (MN752212), o šios tripanosomos tipinis šeimininkas – baltauodegis elnias (*Odocoileus virginianus*, Ruminantia, Cervidae) (Garcia ir kt., 2020).

Neseniai Kostygov su kolegomis (2022) aptiko nežinomą monokseninį tripanosomatidą, žiemojančiuose *Culex torrentium* uoduose. Mūsų tyrime dviejose žiemojančiose *C. pipiens* patelėse buvo aptikti nežinomi tripanosomatidai, kurie 99,87 ir 99,51% panašūs į 2022-aisiais metais Kostygov ir kolegų aptiktą *C. torrentium* hibernuojančiose patelėse. Nepaisant to, kad per pastaruosius du dešimtmečius beveik padvigubėjo žinomų monokseninių tripanosomatidų genčių skaičius, vis dar trūksta informacijos apie tripanosomatidų įvairovę skirtingose vabzdžių grupėse. Nors monokseniniai tripanosomatidai paprastai laikomi nepatogeniniais, yra tyrimų, kurių rezultatai rodo, jog jie gali neigiamai paveikti vabzdžius (Frolov ir kt., 2021), bet tai buvo ištirta tik su keliomis vabzdžių rūšimis. Šie vabzdžių parazitai nėra tiesiogiai susiję su mityba krauju ir gali užkrėsti vabzdžius

įvairiais būdais (pvz., vabzdžiams maitinantis užkrėstu grobiu ar išmatomis arba per užterštus substratus) (Frolov ir kt., 2021; Votýpka ir kt., 2021).

Manome, jog būtų reikalinga tęsti tyrimus, atlikti eksperimentus, siekiant nustatyti tikrųjų uodų gebėjimą peržiemoti užsikrėtus paukščių kraujo parazitais ar monokseniniais tripanosomatidais, kadangi duomenų šioje srityje nėra daug.

Smulkiųjų mašalų užsikrėtimas Haemosporida paukščių kraujo parazitais

2022–2023 metais buvo ištirtos 2 532 bent kartą krauju pasimaitinusios (matomas burgundiškas pigmentas) *Culicoides* patelės. Nustatyta 13-a rūšių, iš kurių gausiausios – *Culicoides obsoletus* rūšių grupė (25,6%), *C. pictipennis* (23,4%), *C. kibunensis* (22,3%) bei *C. festivipennis* (10,7%) (lentelė 3.4.1.1.).

Bendras hemosporidinių parazitų aptikimo ekstensyvumas smulkiuosiuose mašaluose siekė 7,4%. Tyrimo metu keturiuose mašaluose buvo aptikta *Plasmodium* spp. parazitų DNR, *Haemoproteus* spp. parazitų DNR buvo aptikta 177 mašaluose, o dar devyniuose smulkiuosiuose mašaluose aptiktos mišrios infekcijos. Iš viso buvo nustatytos 26-ios *Haemoproteus* genetinės linijos, iš kurių šešios (hCULKIB02, hCULKIB03, hCULKIB04, hCULKIB05, hCULPIC01, hCULPIC02) nustatytos pirmą kartą. Taip pat buvo nustatytos trys *Plasmodium* spp. genetinės linijos iš kurių dvi (pCULFES01, pCULOBS01) buvo aptiktos pirmą kartą (lentelė 3.4.1.1.).

Mikroskopuojant 60-yje smulkiųjų mašalų seilių liaukų mėginių (tai sudaro beveik trečdalį visų PGR teigiamų mėginių) buvo aptikti *Haemoproteus* spp. sporozoitai (lentelė 3.4.1.1.). Pirmą kartą *C. segnis* seilių liaukose rasti *H. fringillae* hCCF3, *H. majoris* hPHSIB1, *H. asymmetricus* hTUPHI01 (3.4.1.2.n pav.) ir *H. minutus* hTURDUS2 sporozoitai (3.4.1.2.q pav.). Taip pat pirmą kartą *C. kibunensis* seilių liaukose rasti *H. belopolskyi* hHIICT1, *H. homominutus* hCUKI1 (3.4.1.2.b pav.), *H. parabelopolskyi* hSYAT01 (3.4.1.2.f pav.) ir hSYAT02 (3.4.1.2.h pav.) bei *Haemoproteus* sp. hSYAT13 sporozoitai (3.4.1.2.j pav.). Pirmą kartą *C. reconditus* rūšies mašalo seilių liaukose aptikti *H. magnus* hROFI1 sporozoitai (3.4.1.2.e pav.). Tuo tarpu *C. pictipennis* seilių liaukose rasti *H. parabelopolskyi* hSYAT01 (3.4.1.2.g pav.), *Haemoproteus* sp. hSYAT13 (3.4.1.2.k pav.), *H. homogeneae* hSYAT16 (3.4.1.2.d pav.), *H. asymmetricus* hTUPHI01 (3.4.1.2.m pav.) ir *H. minutus* hTURDUS2 (3.4.1.2.p pav.) sporozoitai, c *C. festivipennis* - *H. belopolskyi* hHIICT1 (3.4.1.2.a pav.). Genetinių linijų hCUKI1, hROFI1, hSYAT16, hSYAT01, hCULPIC02 (3.4.1.2.c pav.) ir hSYAT13 (3.4.1.2.j,k

pav.) sporozoitai moksliniuose tyrimuose aptinkami pirmą kartą. *Plasmodium* spp. sporozoitai mikroskopuojant nebuvo aptikti.

Sąveikų tinklo analizė parodė sudėtingus ryšius tarp parazitų rūšių bei genetinių linijų ir smulkiųjų mašalų rūšių (3.4.1.3., 3.4.1.4. pav.). Nemažai *Culicoides* rūšių turi sąveikas su keliomis genetinėmis linijomis (*C. kibunensis* – 23; *C. segnis* – 16; *C. obsoletus* grupė – 11; *C. pictipennis* – 11), o tai parodo, jog smulkieji mašalai yra labiau generalistai kraujo parazitų genetinių linijų kontekste.

Diskusija

Bendras hemosporidinių parazitų aptikimo ekstensyvumas smulkiuosiuose mašaluose siekė 7,4%, šis rezultatas yra panašus į ankstesniuose tyrimuose Lietuvoje gautus rezultatus (Bernotienė ir kt., 2019 – 7,0%; Žiegytė ir kt., 2022 – 5,8%). Nors mūsų aptiktas užsikrėtimo ekstensyvumas yra didesnis nei nustatytasis Bulgarijoje (2,0%) (Bobeva ir kt., 2013, 2014) ir Kaliningrade (1,7%) (Bernotienė ir Valkiūnas, 2016), tačiau mažesnis nei Ispanijoje (13,4%) (Ferraguti ir kt., 2013b). Šie skirtumai gali atspindėti tyrimo vietų, vabzdžių gausumo, rūšių įvairovės, maitinimosi pasirinkimų, paukščių rūšių įvairovės, parazitų paplitimo paukščių populiacijose ir tyrimo laiko skirtumus.

Culicoides obsoletus grupės mašalai šiame tyrime buvo gausiausi, o parazitų aptikimo ekstensyvumas juose buvo žemas (0,9%). Ankstesnis tyrimas vienoje iš mūsų mėginių rinkimo vietovių (Verkių regioniniame parke) *C. obsoletus* grupės mašaluose hemosporidinių parazitų DNR neaptiko (Žiegytė ir kt., 2021), tačiau kito tyrimo metu toje pačioje vietovėje 2016 metais buvo nustatytas 6,0% parazitų ekstensyvumas (Bernotienė ir kt., 2019), panašūs rezultatai gauti ir Kuršių Nerijoje (Žiegytė ir kt., 2022). Tai parodo, kokios dinamiškos gali būti šios infekcijos, kurios gali skirtis priklausomai nuo tyrimo vietos ir sezono, o tai tik dar labiau apsunkina šeimininko, parazito ir vektoriaus santykių supratimą. Nepaisant to, jog yra manoma, kad *C. obsoletus* mašalams būdingas mamofilinis mitybos būdas, mūsų aptiktų hemosporidinių parazitų rūšys parodo, jog šios rūšies mašalai taip pat gali maitintis ir paukščių krauju (Bartsch ir kt., 2009; Pettersson ir kt., 2013). Dabartiniame tyrime, kaip ir kitų autorių ankstesniuose tyrimuose *Haemoproteus* spp. sporozoitų *C. obcoletus* grupės smulkiųjų mašalų seilių liaukose nebuvo aptikta.

Įdomu, kad 2021 metais *C. pictipennis* buvo viena iš rečiausiai aptinkamų rūšių (Chagas ir kt., 2022), tačiau 2022 metais ji buvo pati

gausiausia (3.4.1.1. lentelė), o bendras gausumas siekė 23,4%. Tokį neatitikimą galėjo lemti skirtingos medžiagos rinkimo datos: 2021 m. smulkiųjų mašalų rinkimas prasidėjo birželio pabaigoje, o 2022 m. – birželio pradžioje, kai šios rūšies vabzdžiai yra dažniau aptinkami. Taip pat galimi vabzdžių populiacijų svyravimai, ypač vidutinio klimato zonose, kur vabzdžių gausumo pokyčiai yra ryškesni, o tam daug įtakos turi ir klimato sąlygų svyravimai (Bernotienė ir kt., 2021; Cuéllar ir kt., 2018; Mullen ir Murphree, 2019; Saroya ir kt., 2021). Šiame tyrime *C. pictipennis* hemosporidinių parazitų aptikimo ekstensyvumas buvo 16,2 %, o kituose tyrimuose jis svyruoja nuo 4,8 % iki 18,9 % (Bernotienė ir kt., 2019; Žiegytė ir kt., 2021, 2022).

Trečioji pagal aptiktų hemosporidinių parazitų ekstensyvumą rūšis – *C. kibunensis* (10,4%), ankstesniuose tyrimuose Lietuvoje turėjo labai skirtingą ekstensyvumą: 4,5% (Bernotienė ir kt., 2019) ir 45,5% Vilniuje (Žiegytė ir kt., 2021), Kuršių nerijoje – 7,8% (Žiegytė ir kt., 2022), o Čekijoje ekstensyvumas siekė 51,0 % (Synek ir kt., 2013). Tačiau svarbu paminėti, jog toks didelis ekstensyvumo procentas Čekijoje vykdytuose tyrimuose galimai yra dėl to, jog mašalai buvo tiriami sutelktiniuose mėginiuose po kelis individus. Mūsų tyrimų metu buvo ne tik aptikta parazitų DNR *C. kibunensis* smulkiuosiuose mašaluose, bet tiriant šių mašalų seilių liaukų preparatus buvo aptikti *Haemoproteus belopolskyi* hHIICT1, *H. homominutus* hCUKI1, *H. parabelopolskyi* hSYAT01, hSYAT02 ir *Haemoproteus* sp. hSYAT13 sporozoitai (3.4.1.1. lentelė, 3.4.1.2 pav.), o tai reiškia, jog ši smulkiųjų mašalų rūšis yra galimas natūralus šių *Haemoproteus* rūšių vektorius. *Culicoides kibunensis* rūšis jau buvo žinoma kaip natūralus *H. pallidus* hPFC1, *H. minutus* hTURDUS2 ir *H. asymmetricus* hTUPHI01 galimas vektorius (Bernotienė ir kt., 2019; Žiegytė ir kt., 2021).

Ankstesniuose tyrimuose buvo nustatyta, kad kitoje mūsų tyrime svarbioje rūšyje *Culicoides festivipennis*, taip pat buvo aptikta *Haemoproteus* DNR (Bernotienė ir kt., 2019; Bobeva ir kt., 2014; Synek ir kt., 2013; Žiegytė ir kt., 2022). Nors sporozoitai niekada nebuvo aptikti šios rūšies smulkiųjų mašalų seilių liaukų preparatuose, *C. festivipennis* dėl savo ornitofilinės mitybos galimai yra natūralus *Haemoproteus* spp. parazitų pernešėjas (Pettersson ir kt., 2013). Mažas hemosporidinių parazitų aptikimo dažnis šioje rūšyje (Bernotienė ir kt., 2019; Bernotienė ir Valkiūnas, 2016; Bobeva ir kt., 2013, 2014; Žiegytė ir kt., 2021, 2022) rodo, kad norint tiksliau nustatyti *C. festivipennis* vaidmenį pernešant hemosporidinius parazitus, reikalinga atlikti išsamesnius tyrimus. Šiame tyrime *H. belopolskyi* hHIICT1 sporozoitai buvo aptikti *C. festivipennis* seilių liaukose (3.4.1.2.a pav.).

Nors *C. segnis* nebuvo viena iš gausiausių rūšių, tačiau mūsų tyrime juose rasta daug PGR teigiamų mėginių ir nemažai sporozoitų seilių liaukų preparatuose (3.4.1.1. lentelė). Nustatytas ekstensyvumas panašus į anksčiau skelbtą Kuršių nerijoje (Žiegytė ir kt., 2022). Iki šiol *C. segnis* rūšyje buvo aptikta *Haemoproteus* spp. DNR Lietuvoje ir Čekijoje (Synek ir kt., 2013; Žiegytė ir kt., 2021, 2022), o neseniai šios rūšies mašaluose aptikti *H. majoris* hCCF5 ir *H. tartakovskyi* hHAWF1 sporozoitai (Žiegytė ir kt., 2022). Mūsų tyrimas išplečia šį sąrašą įtraukdamas dar keturias *Haemoproteus* rūšis ir genetines linijas: *H. minutus* hTURDUS2, *H. asymmetricus* hTUPHI01, *H. fringillae* hCCF3 ir *H. majoris* hPHSIB1.

Pirmą kartą *C. reconditus* mašaluose aptikta hemosporidinių parazitų DNR bei rasti *Haemoproteus* sporozoitai seilių liaukose. Nors ši rūšis plačiai paplitusi Europoje (Mathieu ir kt., 2012), Lietuvoje aptinkama nedažnai (Bernotienė ir kt., 2019; Chagas ir kt., 2022; Žiegytė ir kt., 2021). Nepaisant mažesnio šios rūšies gausumo Lietuvoje, šis tyrimas patvirtina jos galimą dalyvavimą *Haemoproteus* pernešime.

Sąveikų tinklo analizė parodė sudėtingus ryšius tarp parazitų ir jų vektorių (3.4.1.3., 3.4.1.4. pav.). *Culicoides kibunensis* išsiskiria reikšmingu vaidmeniu *Haemoproteus* spp. parazitų cikluose Europoje. Ši rūšis sąveikauja su 23-jomis *Haemoproteus* spp. genetinėmis linijomis, o tai šiame tyrime yra didžiausia nustatyta sąveikų įvairovė. Sąveikų tinklas parodė, kad *C. kibunensis* gamtoje dažnai sąveikauja su *H. asymmetricus* hTUPHI01 ir *H. minutus* hTURDUS2, kurios yra susijusios su *Turdus merula* ir *T. philomelos*. Genetinė linija hTUPHI01 taip pat turi daug sąveikų ir su *C. segnis* (3.4.1.3., 3.4.1.4. pav.). *Culicoides segnis* sąveikų tinklas yra platus ir ši mašalų rūšis sąveikauja su 16-a *Haemoproteus* spp. genetinių linijų. Tai parodo, jog ši rūšis atlieka svarbų vaidmenį *Haemoproteus* parazitų cikluose. *Culicoides punctatus* dažniausiai sąveikauja su *H. majoris* hPARUS1. *Haemoproteus minutus* hTURDUS2 ir parodo menką specifiškumą vektoriams, kadangi tyrimo metu buvo nustatytos sąveikos su devyniomis rūšimis (3.4.1.3., 3.4.1.4. pav.). Be to, buvo nustatyta, kad *C. circumscriptus* daug sąveikauja su įvairiomis *Haemoproteus* rūšimis (iš viso 11-a sąveikų). Nors mūsų tyrime pavyko surinkti tik tris šios rūšies smulkiuosius mašalus, tai galėjo būti siejama su tuo, kad kai kurios *Culicoides* rūšys renkasi skirtingus buveinių aukščius (Pettersson ir kt., 2013; Service, 1971). Kituose tyrimuose *C. circumscriptus* mėginiai buvo renkami 20–26 metrų aukštyje virš žemės (Braverman ir Linley, 1993; Černý ir kt., 2011). Gauti sąveikų tinklo analizės rezultatai parodo, jog smulkieji mašalai yra labiau generalistai kraujo parazitų genetinių linijų kontekste.

VU Kairėnų botanikos sode buvo aptikta daugiausiai tiek *Haemoproteus* spp. genetinių linijų, tiek *Culicoides* rūšių. Tai gali būti siejama su įvairiomis mikrobuveinėmis, įskaitant lapuočių ir spygliuočių miškelius, įvairius krūmynus, tiek natūraliai išaugusius, tiek susodintus augalus, tvenkinius, mažus upelius, aprūpinančius dirvožemį drėgme, pernykščių lapų sankaupas ir maistinių medžiagų turtingą dirvą – idealias sąlygas *Culicoides* rūšių veisimuisi. VU Kairėnų botanikos sode buvo aptikta daugiausiai burgundišką pigmentą turinčių patelių taip pat aptikta *C. circumscriptus* (rasta tik VU Kairėnų botanikos sode), ir daugiau nei kitose vietovėse aptikta šių rūšių mašalų – *C. festivipennis, C. kibunensis, C. pallidicornis* ir *C. segnis*. VU Kairėnų botanikos sode buvo nustatyta didžiausia *Haemoproteus* spp. genetinių linijų įvairovė – 13-a iš 26-ių buvo aptikta tik šioje vietoje (3.4.2.1. lentelė). Ši didelė įvairovė gali būti susijusi su specifine šeimininkų ir parazitų sąveika. Pavyzdžiui, *Haemoproteus* sp. hSYAT13 ir *H. homogeneae* hSYAT16 anksčiau buvo aptikta tik *Sylvia atricapilla,* o *H. homominutus* hCUKI1 buvo pastebėta *T. philomelos* ir *T. viscivorus* (Valkiūnas ir kt., 2019). Tai leidžia manyti, kad šios paukščių rūšys VU Kairėnų botanikos sode buvo labiau paplitusios, lyginant su kitomis tyrimo vietomis.

Haemoproteus majoris hCWT4 ir *H. magnus* hROFI1 buvo aptiktos tik Puvočių kaime (3.4.2.1. lentelė). Abi linijos aptiktos daugybėje paukščių rūšių (iš viso 21-oje), todėl sunku nustatyti konkretų šeimininką, kurio krauju *Culicoides* mašalai galėjo pasimaitinti (http://130.235.244.92/MalAvi/; informacija gauta 2024 balandžio 24). *Haemoproteus* sp. hCIRCUM05 rastas tik Brinkiškių kaime ir yra žinoma, kad užkrečia tik Corvidae (varniniai) paukščių rūšis (http://130.235.244.92/MalAvi/; informacija gauta 2024 balandžio 24). Corvidae paukščiai yra sėkmingai gyvenantys miesto ir antropogeninės įtakos paveiktoje aplinkoje (Benmazouz ir kt., 2021), o tai gali paaiškinti šio parazito buvimą Brinkiškių kaime. Panašiai *H. syrnii* hCULKIB01, apie kurią pranešta keliose Strigiformes (pelėdiniai) rūšyse (http://130.235.244.92/MalAvi/; informacija gauta 2024 balandžio 24). Ši genetinė linija buvo užfiksuota tik Verkių regioniniame parke, o tai rodo, kad šis parkas galimai turi tinkamesnes buveines Strigiformes paukščiams, lyginant su kitomis tyrimų vietovėmis.

Haemoproteus asymmetricus hTUPHI01 ir *H. minutus* hTURDUS2 genetinės linijos buvo rastos visose tyrimų vietose bei buvo dažniausiai aptiktos smulkiuosiuose mašaluose (lentelės 3.4.1.1., 3.4.2.1.). Šios genetinės linijos dažniausiai aptinkamos *Turdus philomelos* ir *T. merula* (Valkiūnas, 2005; Valkiūnas ir kt., 2021), o didelis jų paplitimas visose vietose gali būti

dėl plataus stuburinių šeimininkų paplitimo. Šių rūšių paukščių taip pat gausu Lietuvoje pavasarį ir vasarą, o jų populiacijos didėja (IUCN, 2023).

Nors buvo aptiktos 26-ios parazitų genetinės linijos, mechanizmai, darantys įtaką šių parazitų plitimui gamtoje, lieka nevisiškai suprasti. Tačiau akivaizdu, kad *C. kibunensis, C. pictipennis* ir *C. segnis* yra reikšmingi *Haemoproteus* spp. paplitime Lietuvoje dėl savo gausos ir ornitofilinių mitybos įpročių (Bobeva ir kt., 2015; González ir kt., 2022; Tomazatos ir kt. , 2020). Tolimesniuose tyrimuose reikia ir toliau tirti šeimininkų ir vektorių biologiją, jų sąveikas tam, kad geriau suprastume jų vaidmenį perduodant *Haemoproteus* parazitus.

IŠVADOS

1. Tikrųjų uodų lervos vandens telkiniuose buvo aptinkamos nuo kovo iki rugsėjo mėn., o didžiausia rūšių įvairovė ir gausumas Lietuvoje užfiksuotas balandžio ir gegužės mėnesiais. Nustatyta, kad vandens telkinių laikinumas, dugno padengimas, pH lygis, nitratų kiekis ir sezoniškumas turi įtakos tam tikrų uodų lervų rūšių buvimui ir gausumui.
2. *Coquillettidia richiardii, Culex pipiens* ir *Culiseta* genties rūšių tikrųjų uodų užsikrėtimo hemosporidiniais parazitais ekstensyvumas buvo didžiausias, todėl šių rūšių tikrieji uodai yra svarbūs hemosporidinių parazitų tyrimų objektai. *Ochlerotatus* rūšių tikrųjų uodų užsikrėtimo hemosporidiniais parazitais ekstensyvumas – mažiausias. Didžiausias ekstensyvumas – nuo liepos iki rugpjūčio. Tikruosiuose uoduose aptiktos penkios *Plasmodium* ir trys *Haemoproteus* rūšys. Ištirtuose tikruosiuose uoduose buvo aptiktos įvairios mišrios infekcijos *Plasmodium, Haemoproteus* ir *Leucocytozoon* genčių parazitais.
3. Buvo nustatyta, jog *Culex pipiens* uoduose *Plasmodium matutinum* (pLINN1) užbaigia sporogoninį vystymąsi, todėl šie uodai yra galimi natūralus vektoriai.
4. Aktyviuose *Culex pipiens* uoduose aptiktos trys *Trypanosoma* genčiai priklausančios rūšys. Hibernuojantys *C. pipiens* uodai buvo užsikrėtę tik monokseniniais tripanosomatidais, o hemosporidinių parazitų juose neaptikta. Tai rodo, jog žiemojantys *C. pipiens* uodai, nėra svarbūs kaip šių parazitų rezervuarai žiemos metu.
5. Hemosporidinių parazitų, priskiriamų genetinėms linijoms hCUKI1, hCULPIC02, hROFI1, hSYAT01, hSYAT13, hSYAT16, sporozoitai

pirmą kartą aptikti smulkiųjų mašalų seilių liaukose. Sporozoitai, priskiriami penkioms genetinėms linijoms (hCUKI1, hHIICT1, hSYAT01, hSYAT13, hSYAT16), pirmą kartą aptikti *C. kibunensis*, penkioms (hSYAT01, hSYAT13, hSYAT16, hTUPHI01, hTURDUS2) – *C. pictipennis*, keturioms (hCCF3, hPHSIB1, hTUPHI01, hTURDUS2) – *C. segnis* ir vienai (hHIICT1) – *C. festivipennis* smulkiųjų mašalų seilių liaukose. Šios smulkiųjų mašalų rūšys yra galimi prieš tai paminėtų genetinių linijų natūralūs vektoriai.

6. Aštuonių iš 13 *Culicoides* rūšių smulkiuosiuose mašaluose buvo aptikta 11 *Haemoproteus* ir *Plasmodium* rūšių DNR (29 genetinės linijos), tai rodo šių *Culicoides* smulkiųjų mašalų rūšių ornitofilino maitinimosi įpročius.
7. *Culicoides reconditus* smulkiuosiuose mašaluose *Haemoproteus* hemosporidiniai parazitai (*H. magnus* hROFI1) geba užbaigti sporogoniją ir todėl šios rūšies smulkieji mašalai yra galimi natūralūs šių patogenų pernešėjai.

ACKNOWLEDGMENTS

First and most importantly I would like to sincerely thank my supervisor dr. Rasa Bernotienė, who accepted me as her first PhD student, was always helpful, had critical insight and helped me to move on with the work. I hope that in the future we can work hand in hand as colleagues.

I would like to thank the reviewers for all their time and efforts in improving this dissertation. Your expert insights are highly valued.

I am happy to have had the pleasure of working with wonderful colleagues from Entomology Laboratory. My topic required close collaboration with other laboratories, so I would like to thank P.B. Šivickis Laboratory of Parasitology as well as Laboratory of Molecular Ecology and Laboratory of Chemical and Behavioral Ecology for not feeling like a stranger.

It was pleasant and helpful working with VU student Gabrielė Kalinauskaitė. It was a pleasure teaching her as well as working with her.

Thank you to VU Kairėnai Botanical Garden staff for all the help and for letting us conduct the research in the garden. Collected material was important and significant for this work.

I would like to thank my husband Tomas Pocius who always supported me not only emotionally but also never left me alone when I needed help and helped in material collection, search of research location whenever he could. Thank you.

Thank you to Almantas Kulbis for helping in research locations search, support and productive discussions.

Finally – thanks to my family and friends. Your support means the world to me.

LIST OF PUBLICATION BY THE AUTHOR ON THE DISSERTATION TOPIC

I. Chagas, C.R.F.; Hernandez-Lara, C.; Duc, M.; **Valavičiūtė-Pocienė, K.**; Bernotienė, R. 2022. What Can Haemosporidian Lineages Found in *Culicoides* Biting Midges Tell Us About Their Feeding Preference? *Diversity*, *14*: 957. https://doi.org/10.3390.d14110957

II. Chagas, C.R.F.; Duc, M.; Kazak, M.; **Valavičiūtė-Pocienė, K**.; Bukauskaitė, D.; Hernández-Lara, C.; Bernotienė, R. 2024. High Abundance of *Haemoproteus* Parasites in *Culicoides* (Diptera, Ceratopogonidae), with a Confirmation of *Culicoides reconditus* as a New Vector of These Avian Blood Parasites. *Insects*, *15*: 157. https://doi.org/10.3390/insects15030157

III. **Valavičiūtė-Pocienė, K**.; Kalinauskaitė, G.; Chagas, C.R.F.; Bernotienė, R. 2024. Avian haemosporidian parasites from wild-caught mosquitoes with new evidence on vectors of *Plasmodium matutinum*. *Acta Tropica*, *256*: 107260. https://doi.org/10.1016/j.actatropica.2024.107260

IV. **Valavičiūtė-Pocienė, K.**; Bernotienė, R. 2024. Survey on mosquito larvae (Diptera: Culicidae) in different water bodies in Lithuania. *Bulletin of Insectology* (accepted)

V. **Valavičiūtė-Pocienė K.**; Kazak M.; Ježova T.; Kalinauskaitė G.; Bernotienė R. 2024. Investigation of blood parasites (Haemosporida, Trypanosomatida) in hibernating *Culex* mosquitoes. *Molecular Research*: *15*(4), 2184-2198. https://doi.org/10.3390/microbiolres15040146

AUTHOR CONTRIBUTIONS IN THE CORRESPONDING PAPERS

I. Conceptualization, C.R.F.C.; methodology, R.B.; formal analysis, C.R.F.C. and C.H.-L.; investigation, C.R.F.C., C.H.-L., M.D., **K.V.-P**., and R.B.; resources, C.R.F.C. and R.B.; data curation, C.R.F.C. and C.H.-L.; writing—original draft preparation, C.R.F.C. and C.H.-L.; writing—review and editing, C.R.F.C., C.H.-L., M.D., **K.V.-P**., and R.B.; supervision, C.R.F.C.; pro- ject administration, C.R.F.C.; funding acquisition, C.R.F.C.

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LIST OF CONFERENCE PRESENTATIONS BY THE AUTHOR ON THE SUBJECT OF THE DISSERTATION

1. **Valavičiūtė-Pocienė K.**, Bernotienė R. 2021. *Culicoides* biting midges: species composition, flying activity and intraspecific diversity - *9th Conference of the Scandinavian--Baltic Society for Parasitology*, April 21-23, Vilnius, Lithuania.
2. Chagas C. R. F., Hernández-Lara C., Duc M., **Valavičiūtė-Pocienė K.**, Bernotienė R. 2022 Identification of natural vectors of *Haemoproteus* parasites - *15th International Congress of Parasitology,* August 21-26, Kopenhaga, Danija.
3. **Valavičiūtė-Pocienė K.,** Bernotienė R. 2022. Seasonal activity of mosquitoes in Lithuania with some notes on their role as vectors of avian malaria – *5th International Conference on Malaria and Related Haemosporidian Parasites of Wildlife*, September 5-8, Bielefeld, Vokietija. Book of abstracts: 131
4. **Valavičiūtė-Pocienė K.**, Bernotienė R. 2023. Peculiarities of mosquito larvae distribution in different water bodies - The 66th international conference OPEN READINGS, April 18-21, Vilnius.
5. **Valavičiūtė-Pocienė K**., Bernotienė R. 2023. Haemosporidae parasites in wild-caught *Coquillettidia* and *Culex* mosquitoes - 10th Conference of the Scandinavian-Baltic Society for Parasitology, June 5-7, Tartu, Estija.
6. **Valavičiūtė-Pocienė K**., Bernotienė R. 2023. The impact of various parameters of water bodies for the abundance and species composition of mosquito larvae - XI international EMCA conference, October 7-10, Palma de Mallorca, Ispanija.

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. 3rd place for the best Poster presentation at the XI international EMCA conference, Palma de Mallorca, Spain.

CURRICULUM VITAE

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Education and Academic Degrees:

2020-2024 PhD, Ecology and Environmental Sciences, Nature Research Centre and Vilnius University, Vilnius, Lithuania

2016-2018 MSc, Biological Diversity, Vilnius University, Vilnius, Lithuania

2012-2016 BSc, Biology, Vilnius University, Vilnius, Lithuania

Work experience

2017-2020 Junior Environmental Expert, Baltic Environmental Forum, Vilnius, Lithuania

2021-2024 Junior Researcher, project "METAH", Nature Research Centre, Vilnius, Lithuania

2021-2022 Biologist, project "DIVAKS", Nature Research Centre, Vilnius, Lithuania

2023 Lecturer, Life Sciences Center, Vilnius University, Vilnius, Lithuania

2024 Biologist, project „VECTOR", Nature Research Centre, Vilnius, Lithuania

Main Research Area

The role of blood-sucking (Culicidae) mosquitoes and biting midges (Ceratopogonidae) in the transmission of avian blood parasites (Haemosporida, Trypanosomatida). Biology and ecology of blood-sucking mosquitoes.

Internships

2022 11-28 / 12-04. Practice of bloodsucking insect identification, material collecting and storing methods (University of Balearic Islands (UIB), Palma de Mallorca, Spain).

Language Proficiency

Lithuanian (native), English (fluent), Russian (moderate).

Bachelor Students Supervision

2022 – 2024 Scientific consultant for Gabrielė Kalinauskaitė, Biology.

Scientific Publications

Valavičiūtė K., Fernandes Chagas C. R., Bernotienė R. 2020. Data on the biting midges of the genus *Culicoides* Latreille (Diptera: Ceratopogonidae) in Labanoras Regional Park (Eastern Lithuania). *Bulletin of the Lithuanian Entomological Society, 4* (32), 120–124.

Markevičiūtė R., **Valavičiūtė-Pocienė K.,** Rintala T. 2021. *Heterogaster artemisiae* Schilling, 1829 (Heteroptera: Heterogastridae) a new species for Lithuania. *Bulletin of the Lithuanian Entomological Society*, *5* (33), 20–22.

Chagas, C.R.F., Hernandez-Lara, C., Duc, M., **Valavičiūtė-Pocienė, K.,** Bernotienė, R. 2022. What Can Haemosporidian lineages found in *Culicoides* biting midges tell us about their feeding preferences? *Diversity*, *14*(11): 957.

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Valavičiūtė-Pocienė, K.; Kalinauskaitė, G.; Chagas, C.R.F.; Bernotienė, R. 2024. Avian haemosporidian parasites from wild-caught mosquitoes with new evidence on vectors of *Plasmodium matutinum*. *Acta Tropica*, *256*: 107260.

Valavičiūtė-Pocienė, K.; Bernotienė, R. 2024. Survey on mosquito larvae (Diptera: Culicidae) in different water bodies in Lithuania. *Bulletin of Insectology* (accepted)

Kazak, M.; **Valavičiūtė-Pocienė, K.;** Kondrotaitė, S.; Duc, M.; Bukauskaitė, D.; Hernández-Lara, C.; Bernotienė, R.; Chagas C.R.F. 2024. *Culicoides* biting midges feeding behaviour as a key for understanding avian *Haemoproteus* transmission in Lithuania. *Medical and Veterinary Entomology*, *38*(4): 530-541. https://doi.org/10.1111/mve.12752

Valavičiūtė-Pocienė K.; Kazak M.; Ježova T.; Kalinauskaitė G.; Bernotienė R. 2024. Investigation of blood parasites (Haemosporida, Trypanosomatida) in hibernating Culex mosquitoes. *Molecular Research*: *15*(4), 2184-2198.
https://doi.org/10.3390/microbiolres15040146

Scientific Conferences

Valavičiūtė-Pocienė K., Bernotienė R. 2021. *Culicoides* biting midges: species composition, flying activity and intraspecific diversity - 9th Conference of the Scandinavian--Baltic Society for Parasitology, balandžio 21-23, Vilnius, Lithuania.

Chagas C. R. F., Hernández-Lara C., Duc M., **Valavičiūtė-Pocienė K.,** Bernotienė R. 2022 Identification of natural vectors of *Haemoproteus* parasites - 15th International Congress of Parasitology, rugpjūčio 21-26, Copenhagen, Denmark.

Valavičiūtė-Pocienė K., Bernotienė R. 2022. Seasonal activity of mosquitoes in Lithuania with some notes on their role as vectors of avian

malaria – 5th International Conference on Malaria and Related Haemosporidian Parasites of Wildlife, rugsėjo 5-8, Bielefeld, Germany. Book of abstracts: 131

Valavičiūtė-Pocienė K., Bernotienė R. 2023. Peculiarities of mosquito larvae distribution in different water bodies - The 66th international conference OPEN READINGS, balandžio 18-21, Vilnius, Lithuania.

Valavičiūtė-Pocienė K., Bernotienė R. 2023. *Haemosporidae* parasites in wild-caught Coquillettidia and Culex mosquitoes - 10th Conference of the Scandinavian-Baltic Society for Parasitology, birželio 5-7, Tartu, Estonia.

Valavičiūtė-Pocienė K., Bernotienė R. 2023. The impact of various parameters of water bodies for the abundance and species composition of mosquito larvae - XI international EMCA conference, spalio 7-10, Palma de Mallorca, Spain.

External Professional Activities

2021 – Present Member of the Scandinavian – Baltic Society for parasitology

2021 – Present Member of Lithuanian Entomological Society

2023 – Present Member of European Mosquito Control Asociation

2023 – Present Participant of Wildlife Malaria Network, COST action

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