Eesti Maaülikool
Estonian University of Life Sciences

MOSQUITO (DIPTERA: CULICIDAE) DIVERSITY IN THE URBAN ENVIRONMENT AND COUNTRYSIDE OF ESTONIA

PISTESÄÄSKLASTE (DIPTERA: CULICIDAE) MITMEKESISUS EESTI LINNAKESKKONNAS JA LOODUSES

HELI KIRIK

A Thesis
for applying for the degree of Doctor of Philosophy
in Applied Biology

Väitekiri
filosoofiadoktori kraadi taotlemiseks
rakendusbioloogia erialal

Tartu 2022

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following research papers, which are referred to in the text by their Roman numerals:

I. **Kirik, Heli;** Tummeleht, Lea; Lilja, Tobias; Kurina, Olavi (2020). Novel mitochondrial DNA lineage found among *Ochlerotatus communis* (De Geer, 1776) of the Nordic-Baltic region. Insects, 11(6), 397. DOI: 10.3390/insects11060397

II. **Kirik, Heli;** Burtin, Viktoria; Tummeleht, Lea; Kurina, Olavi (2021). Friends in all the green spaces: weather dependent changes in urban mosquito (Diptera: Culicidae) abundance and diversity. *Insects*, 12(4), 352. DOI: 10.3390/insects12040352

III. **Kirik, Heli;** Tummeleht, Lea; Kurina, Olavi (2022). Rediscovering the Estonian mosquito (Diptera: Culicidae) fauna: an annotated checklist with illustrations and DNA evidence. *Zootaxa*, 5094(2), 261-287. DOI: 10.11646/zootaxa.5094.2.3

Table 1. The authors' contribution to these papers is as follows:

	I	II	III
Idea and study design	**HK**, LT, OK, TL	OK, VB, LT	OK, LT, **HK**
Sampling	**HK**, LT, OK	**HK**, VB, OK	**HK**, LT, OK
Laboratory work	**HK**	**HK**, VB, OK	**HK**, OK
Data analysis	**HK**, TL, LT	**HK**, LT	**HK**
Visualization	**HK**	**HK**	OK, **HK**
Manuscript preparation	**HK**, OK, LT, TL	**HK**, OK, LT, VB	**HK**, OK, LT

VB – Viktoria Burtin; **HK** – Heli Kirik; OK – Olavi Kurina; TL – Tobias Lilja; LT – Lea Tummeleht.

ABBREVIATIONS

Ae.	genus *Aedes*
An.	genus *Anopheles*
CI	confidence interval
COI	cytochrome c oxidase subunit 1
Cq.	genus *Coquillettidia*
Cs.	genus *Culiseta*
Cx.	genus *Culex*
DMSO	dimethyl sulfoxide
ENS	effective number of species
GLM	generalized linear model
indels	nucleotide insertions and deletions
ITS2	internal transcribed spacer 2
IQR	interquartile range
MBID	mosquito borne infectious disease
mtDNA	mitochondrial deoxyribonucleic acid
ND5	NADH dehydrogenase subunit 5
nDNA	nuclear deoxyribonucleic acid
NML	novel mitochondrial lineage
NuMts	nuclear mitochondrial pseudogenes
PCR	polymerase chain reaction
Q1	lower quartile
Q3	upper quartile
S.E.	standard error
wsp	*Wolbachia* surface protein

1. INTRODUCTION

Mosquitoes (Diptera: Culicidae) are globally important insects (Insecta) that can be found on every continent except Antarctica (Rueda 2008). According to the latest estimation, researchers have described about 3 591 extant mosquito species thus far (Harbach 2021), but the real number of distinct species is probably larger. Although only a relatively small number of mosquitoes are medically significant, a thorough understanding of the diversity and ecology of these dipterans is particularly important, as they have a profound effect on the wellbeing of other organisms.

For the most part, mosquitoes consume nectar and plant liquids for energy and only the females of some species require vertebrate blood as an extra protein source for egg production (Becker *et al.* 2020). Nevertheless, the bites of blood-seeking females can have serious consequences for the victim. Mosquitoes are believed to be the world's most important pathogen vectors – organisms capable of carrying disease agents from one host to another. Vector-borne pathogens are responsible for 17% of the global communicable disease burden, accounting for billions of infections and more than 700 000 human deaths every year (WHO 2014, 2017). These illnesses can cause immense suffering and be a considerable economic burden on individuals as well as whole countries (Athni *et al.* 2021). It is important to note that mosquito-borne infectious diseases (MBIDs) like malaria, dengue, chikungunya, yellow-fever, etc., make up a large part of all vector-borne diseases (WHO 2017). Although MBIDs are disproportionately common in the tropics and subtropics, some of these infections can also occur in colder climates (Evans & Peterson 2019; Deksne *et al.* 2020). Furthermore, frequent insect bites can also influence human and animal welfare through allergic reactions (Cantillo & Puerta 2021) and by discouraging outdoor activities (Worobey *et al.* 2013; Halasa *et al.* 2014). All of these factors make mosquitoes important research subjects, as improved knowledge of the local hematophagous insect fauna will help evaluate potential risks and allow researchers to make more accurate predictions for the future.

Due to their medical and veterinary importance, mosquitoes as well as MBIDs have already been extensively researched. For example, the main anopheline malaria (*Plasmodium* spp.) vectors of different regions,

such as *Anopheles gambiae* Giles in Sub-Saharan Africa and *An. darlingi* Root in South America (Sinka *et al.* 2012), are the focus of many studies. The same applies for particularly invasive species like the yellow fever mosquito *Aedes aegypti* (Linnaeus) and the Asian tiger mosquito *Ae. albopictus* (Skuse), which are now common throughout tropical and subtropical regions (Beebe *et al.* 2013; Kraemer *et al.* 2015). Especially with *Ae. albopictus*, who is expanding its distribution range further into the temperate region (Bonizzoni *et al.* 2013; Bellini *et al.* 2020) and likely continuing to spread even further in the future (Schaffner *et al.* 2009; Fischer *et al.* 2011). Such changes in the distribution of various mosquitoes as well as advances in genetic identification have resulted in many countries updating their local species checklists and implementing continuous mosquito surveillance. Naturally, researchers have also been interested in describing the phylogeny of Culicidae – whether analyzing connections between or within genera (Sallum *et al.* 2002; Reidenbach *et al.* 2009; Reinert *et al.* 2009) or investigating the relationships among particular sister species (Ma *et al.* 2006; Foley *et al.* 2007b; Paredes-Esquivel *et al.* 2009). Understandably, the pathogens responsible for MBIDs (Weissenböck *et al.* 2010; Halbach *et al.* 2017) have been another important avenue of study. Numerous articles have been written concerning the interactions between disease agents and their vectors (Lefèvre *et al.* 2013; Kramer 2016; Neelakanta & Sultana 2016) as well as about the vector competence and capacity of various mosquito species (Balenghien *et al.* 2008; Turell *et al.* 2008; Martinet *et al.* 2019). Likewise, new light has been shed on the way female mosquitoes choose their next blood meal (Raji & DeGennaro 2017; Wynne *et al.* 2020) and on other aspects of mosquito ecology (Chandrasegaran *et al.* 2020). This has led to the development of various repellents (Islam *et al.* 2017; Tavares *et al.* 2018) and control methods (Reis-Castro & Hendrickx 2013; Sicard *et al.* 2019) over the decades. Of course, there are a myriad of other mosquito research topics occupying scientists around the world.

While medical entomology has been an important study subject for a long time, much of the research has understandably focused on tropics and subtropics as well as on the most important species and vector-borne pathogens therein. There are likely still numerous cryptic species to be discovered and gaps in our understanding of the ecology of many species. Furthermore, as local biotic and abiotic factors can have a profound effect on mosquito ecology (Chandrasegaran *et al.* 2020) and different species have varying preferences and tolerances, which can be

subject to microevolution (Suesdek 2019), it is important to study these dipterans in a wide range of contexts. Advances in research techniques, new discoveries as well as global environmental changes have made it apt to provide updated information concerning the mosquito fauna of different regions. For example, there is a large gap in the study of blood-feeding dipterans in Estonia after 1957, when the first mosquito checklist was published for the country. This is in contrast to some neighboring areas, which have received multiple updates to their mosquito checklists during the recent decades.

Due to the negative effects mosquitoes have on their hosts as well as the changes in the global distribution of vectors and vector-borne pathogens, this thesis aims to provide a stronger base for mosquito research in Estonia. To do this, light has been shed on the intraspecific genetic variation within populations of the most common species in the country, while discussing its implications for DNA barcoding (**I**). Research was also done to determine the ways meteorological factors influence mosquito abundance and diversity in urban green spaces, to better understand how future climate change and ongoing urbanization could influence the local mosquito fauna (**II**). Finally, the Estonian mosquito checklist was updated for the first time in more than 60 years (**III**).

2. LITERATURE REVIEW

2.1. Mosquito (Diptera: Culicidae) biology

Mosquitoes are two-winged (Diptera) insects intrinsically connected to both terrestrial and aquatic habitats. While about 3 591 extant mosquito species (Harbach 2021) have been described thus far, their real diversity is likely larger. This is in part due to overlooked cryptic species (Zheng 2020) – taxa that cannot be identified by morphological traits alone.

The mosquito lifecycle consists of an egg stage, four larval instars, pupation and an adult stage. Access to open water is an especially important factor, as mosquito larvae can only develop in aquatic environments (Becker *et al.* 2020). However, different species have various preferences for larval habitats: some favor water-filled containers or temporary puddles, others large permanent waterbodies, with various levels of salinity (Soghigian *et al.* 2017; Chandrasegaran *et al.* 2020). This in turn influences local mosquito abundance and diversity. Furthermore, mosquitoes can survive unfavorable periods, like cold or dry seasons, by entering dormancy (diapaus). Species have developed different strategies for overwintering: some go through diapaus during the egg stage, others as an instar and others still as adults (Becker *et al.* 2020). This variation determines which mosquito species are active at any given time. Mosquitoes can have multiple generations per year, especially in warmer regions where they do not enter diapause. As a rule, individuals of both genders mainly feed on nectar and plant liquids (Barredo & DeGennaro 2020) and only females are capable of taking a blood meal. Host-seeking mosquitoes use vision, olfaction, mechanoreception as well as the acoustic, hydric and thermal gradients to find their prey (Wynne *et al.* 2020). Most mosquitoes only fly a few thousand meters in search of mates or hosts, but some species have been known to actively travel more than 30 km (Becker *et al.* 2020). However, mosquitoes can disperse even further accidentally, through global trade and travel routes (Tatem *et al.* 2006).

Mosquitoes themselves are prey or hosts for many different organisms. Mosquito larvae are an important food source for fish (Osteichthyes), amphibians (Amphibia), crustaceans (Crustacea) and other arthropods (Dambach 2020). However, mosquitoes that are capable of breeding in

small water containers or temporary pools can be relatively protected from aquatic predators (Batzer & Wissinger 1996). Additionally, mosquito imagines are often consumed by birds (Aves) and bats (Chiroptera) as well as some invertebrates, like spiders and dragonflies (Becker *et al.* 2020). Water mite (Hydrachnidia) larvae have been known to parasitize on mosquitoes, using them both as a food source and for dispersal (Jalil & Mitchell 1972; Werblow *et al.* 2015). Previous research has shown that the infected mosquitoes have a reduced flight ability, shorter lifespans, slowed growth and produce less eggs (Lanciani & Boyt 1977; Rajendran & Prasad 1992; Biswas *et al.* 2007). However, to control the population size of mosquitoes, the intercellular bacterium *Wolbachia pipientis* Hertig is far more important. This symbiont is known to induce cytoplasmic incompatibility, parthenogenesis and even feminization in mosquitoes (Correa & Ballard 2016). Furthermore, since *Wolbachia* are maternally inherited and effect the reproductive success of the infected individuals, they can impact the mitochondrial DNA (mtDNA) of the hosts as well (Turelli & Hoffmann 1995; Yeap *et al.* 2016). *Wolbachia* symbionts have also been found to make some mosquito species less likely to transmit vector-borne pathogens, thus protecting humans and animals (Moreira *et al.* 2009). It is widely thought that many vector-borne viruses have evolved from arthropod specific disease agents (Halbach *et al.* 2017).

2.3. Vectors and vector-borne pathogens in a changing world

Mosquitoes influence the wellbeing of others through the discomfort caused by their blood-feeding behavior as well as by transmitting various pathogens. Insect bites have been known to decrease people's enjoyment in outdoors activities (Halasa *et al.* 2014). Also, both mosquito saliva and body allergens can induce several types of allergic reactions in sensitized individuals: from skin reactions to asthma and in some cases even anaphylaxis (Cantillo & Puerta 2021). Most importantly, vector-borne diseases account for about 17% of the global disease burden, with around 80% of the world's population living in risk areas, and pathogens transmitted by mosquitoes are responsible for a large proportion of these infections (WHO 2017). Such illnesses can cause immense suffering as well as considerable economic loss, often affecting poor and already vulnerable social groups more than those whose economic situation is better (UNDP 2017; Athni *et al.* 2021). Unfortunately, vector-borne diseases have become even more prominent during recent decades, due to the emergence as well as re-emergence of

several pathogens (Kilpatrick & Randolph 2012). For example, malaria continues to be an important threat to human health, despite ongoing control efforts (Bhatt *et al.* 2015). Additional diseases like dengue fever, Zika fever, chikungunya fever, yellow fever, West Nile fever and Japanese encephalitis are also major global concerns, having reached far outside of their original endemic regions (Kilpatrick & Randolph 2012; Mayer *et al.* 2017; Huang *et al.* 2019). Consequently, mosquito-borne diseases are becoming increasingly relevant in colder climate zones. The tularemia bacterium *Francisella tularensis* (McCoy & Chapin) as well as the Sindbis virus have been known to cause local outbreaks in the Nordic-Baltic region for a while now (Brummer-Korvenkontio *et al.* 2002; Kurkela *et al.* 2005; Bergqvist *et al.* 2015; Tingström *et al.* 2016). However, the filarial nematode *Dirofilaria repens* Railliet & Henry which is manly a carnivore parasite, but can also infect humans, has recently expanded its range as far north as Finland (Deksne *et al.* 2020).

Mosquitoes can be found almost anywhere in the world, though their species' richness increases towards the equator (Foley *et al.* 2007a). However, processes like urbanization, changes in agricultural practices, deforestation, climate change and socioeconomic developments influence mosquito abundance as well as their geographic ranges and the pathogens they carry (Dhiman & Singh 2017; Franklinos *et al.* 2019; Brugueras *et al.* 2020). For example, due to frequent long distance trade and travel, invasive mosquitoes have become a significant problem around the world (Medlock *et al.* 2012; Kraemer *et al.* 2015). In fact, the spread of the Asian tiger mosquito *Ae. albopictus* was found to correlate well with the most active shipping routes between climatically similar ports (Tatem *et al.* 2006). It has been argued that climate change has not been as important in the distribution changes of vector-borne diseases as the aforementioned factors (Zell *et al.* 2008). However, both mosquito-borne pathogens as well as their vectors are deeply influenced by climatic factors and have been found to respond to weather anomalies, which are projected to become increasingly frequent in the future due to climate change (Semenza & Suk 2018; Brugueras *et al.* 2020; Colón-González *et al.* 2021). Because of this, there have been numerous calls for increased mosquito surveillance in countries around the world.

The composition of the local mosquito fauna can vary noticeably between different habitats, being influenced by the availability of specific aquatic breeding sites, by temperature as well as by many other factors

(Bernotienė 2012; LaDeau *et al.* 2013; Hendy *et al.* 2020). Urban areas make for especially unique environments, often leading to a decline in species' diversity (Jones & Leather 2012). City landscapes tend to be very fragmented, favoring generalists and synanthropic organisms (Faeth *et al.* 2011; Adams *et al.* 2020). Also, urban environments are usually significantly warmer than surrounding areas, which can lead to further changes in the diversity of native arthropods as well as create suitable habitats for organisms that are normally found in warmer climates (Oke 1973; Misslin *et al.* 2016; Youngsteadt *et al.* 2017). Also, cities provide blood-feeding arthropods with easy access to a large amount of hosts, which is especially beneficial for anthropophilic species. It has been theorized that urbanization is one of the main factors for mosquitoes developing a preference for human blood (Rose *et al.* 2020). Urban green spaces are particularly noteworthy sites of human-mosquito contact, which also provide insects with shelter, food plants and numerous breeding sites (Medeiros-Sousa *et al.* 2015, 2017; Zhao *et al.* 2020).

2.4. Culicidae of the Nordic-Baltic region

Several Nordic-Baltic countries have received at least one update to their mosquito checklist during this century, but up to now the first and only Estonian list was published over 60 years ago (Remm 1957). The amount of attention mosquitoes have received in countries neighboring Estonia is markedly varied. For example, 36 mosquitoes were included on a literature based Lithuanian Diptera checklist that was published at the very beginning of the year 2000 and information concerning one additional species was published in 2011 (Pakalniškis *et al.* 2000; Bernotienė & Lučiūnaitė 2011). There was also a separate study looking at the mosquito fauna of the Curonian Spit, which is a sandspit by the Baltic Sea shared by Lithuania and Russia (Bernotienė 2012). Coincidentally, the last Latvian mosquito checklist was also published in 2000. However, it only included 25 taxa, while the true number of mosquito species in the country is likely much higher (Spungis 2000). In comparison, the Finnish mosquito fauna has been reviewed numerous times during the last 10 years and 43 mosquito species have been reported from the country thus far (Huldén & Huldén 2014; Culverwell 2018; Culverwell *et al.* 2020, 2021). The most recent large publication concerning the Swedish mosquito checklist included 49 species (Lundström *et al.* 2013), but the number of taxa reported from the country has now risen to 55 (Möhlmann *et al.* 2017; Robert *et al.* 2019). The currently most

comprehensive list of Norwegian mosquito species was published back at the end of the 20th century (Mehl 1996). However, the mosquitoes of western Russia are relatively well studied, especially over the last few years (Gornostaeva 2000; Khalin & Aibulatov 2020, 2021). Still, many questions concerning the distribution of mosquitoes in Nordic-Baltic countries remain. This is especially true for Estonia.

Genetic identification has been especially important for revising species checklists, as it has now become possible to differentiate between morphologically very similar or even identical taxa. Although mosquitoes are relatively well studied compared to other arthropods, there are still numerous questions remaining, even regarding species delimitation. While growing standardization and lowering costs of DNA barcoding have facilitated many discoveries and helped with mosquito surveillance, there can be problems with using only one marker for species identification. Especially when dealing with mitochondrial sequences. For example, some mosquitoes collected in the Nordic-Baltic region have cytochrome c oxidase subunit 1 (COI) gene sequences more closely related to the North-American species *Ae. tahoensis* Dyar than any of the local taxa. *Aedes tahoensis* belongs to a complex of closely related species, which also contains *Ae. communis* (De Geer), *Ae. churchillensis* Ellis and Burst and *Ae. nevadensis* Chapman & Barr (Brust & Munstermann 1992). Out of these, *Ae. communis* is thus far the only species found in Europe. Additionally, it is difficult to differentiate between these species based on morphological characteristics. In cases like these, multiple genetic markers should be used to get more accurate information regarding the true identity of the problematic specimens.

3. HYPOTHESES AND AIMS OF THE STUDY

Mosquitoes have significant medical and veterinary importance, but they have not been consistently studied in Estonia after the first half of the 20th century. However, a comprehensive understanding of mosquito populations and their general species diversity is becoming increasingly important due to ongoing global changes in the distributions of both insect-borne pathogens as well as the arthropods that carry them. Also, there are still numerous unanswered questions concerning mosquito phylogeny, biology, ecology and many other aspects. This thesis has been undertaken to shed light on the mosquitoes inhabiting Estonia and the factors influencing their abundance and diversity in an urban setting.

The main hypotheses of the study were:

- The widespread species *Ae. communis* exhibits distinct mtDNA lineages.

- Temperature is the driving factor for the abundance of urban mosquitoes.

- There are more mosquito species in Estonia than reported in the original 1957 checklist by Hans Remm.

The aims of the thesis were:

- To determine the intraspecific genetic diversity of *Ae. communis* in the Nordic-Baltic region (**I**).

- Show how various weather factors affect urban mosquito abundance and diversity in the boreal region (**II**).

- Compile an updated Estonian mosquito checklist (**III**).

4. MATERIALS AND METHODS

4.1. Research area and sampling sites

This thesis is based on studies (**I**, **II**, **III**) conducted in Estonia, which is part of the Nordic-Baltic region. Estonia is situated by the Baltic Sea, on the East-European Plain (Raukas 1995) and neighbored by Sweden to the west, Finland to the north, Russia to the east and Latvia to the south. The state of 45 339 km^2 is home to a little over 1.3 million people, making it relatively sparsely populated compared to other European countries (Statistics Estonia 2020; Eurostat 2021). In total, around 85% of the area is made up of forests, agricultural land, bogs and permanent inland water bodies (Environment Agency 2020). The landscape was shaped in large part by the last ice age, when Estonia was covered by the Eurasian Ice Sheet, which receded from the area around 14 000 years ago (Raukas 2009; Patton *et al.* 2017). The area belongs to the warm-summer humid continental climate zone according to the Köppen-Trewartha classification (Kottek *et al.* 2006; Beck *et al.* 2018) and is managed as part of the Boreal Region by the European Commission (Sundseth 2009). Although it depends on the specific year, mosquitoes are generally active from April to October in the country (Remm 1957). Collection sites were located in different areas of Estonia to sample various biomes, while also making it possible for the traps to be supervised and periodically emptied (**I**, **II**, **III**) (Fig. 1). Study sites were used for varying amount of time and during different years, depending on the availability of traps, volunteers and researchers.

A study (**II**, **III**) concentrating on urban mosquitoes took place in Tartu, the second largest town of Estonia. Tartu is a university town and home to almost 100 000 people, located in the South-East region of the country, where lowlands and drumlins are common (Villoslada *et al.* 2017). The town itself is divided by the river Emajõgi and spread out over an area of 38.8 km^2, containing about 3.7 km^2 of public green spaces and 7.1 km^2 of private yards as well as 5.1 km^2 of semi-natural areas (Raud *et al.* 2014). Sampling sites (**II**, **III**) were set up on either side of the river, in frequently used urban green spaces (Fig. 2). These locations included more or less wooded parks and recreational sites as well as the areas of two cemeteries. The sites were arranged to be of various distances from the river, with collection points on both shores

of Emajõgi. Six locations were used during the first study year, one new site was added in the second year and further eight were added at the start of the third collection season. Sites 1 to 6 were visited by V. Burtin in 2013, the same locations plus site 7 were collected from by the author in 2016 and 2017. Sites A to H were visited by T. Kesküla in 2017.

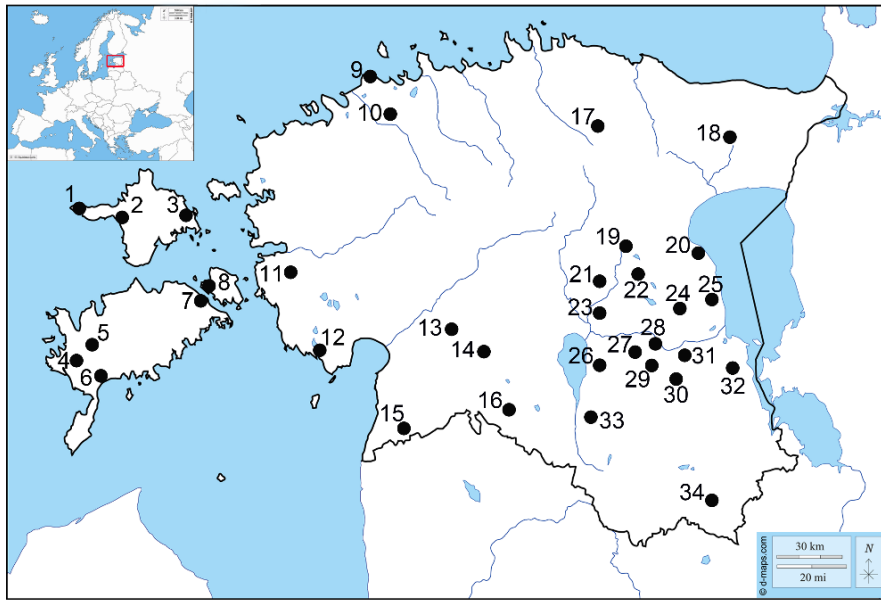

Figure 1: Mosquito collection sites in Estonia: 1 – Kalana, 2 – Vanajõe, 3 –Kerema, 4 – Viidumäe, 5 – Karujärve, 6 – Mändjala, 7 – Orissaare, 8 – Igaküla, 9 – Muraste, 10 – Üksnurme, 11 – Lihula, 12 – Tõstamaa, 13 – Jõesuu, 14 – Punaküla, 15 – Nigula NR, 16 – Viivre, 17 – Lasila, 18 – Mäetaguse, 19 – Kibuvitsa, 20 – Omedu, 21 – Kursi, 22 – Luua, 23 – Laeva, 24 – Undi, 25 – Kolkja, 26 – Maiorg, 27 – Külitse, 28 – Tartu, 29 – Pargi, 30 – Hurda, 31 – Melliste, 32 – Järvselja, 33 – Puka, 34 – Leoski. Base maps of Europe and Estonia: © 2007-2021 https://d-maps.com. Adapted from **III**.

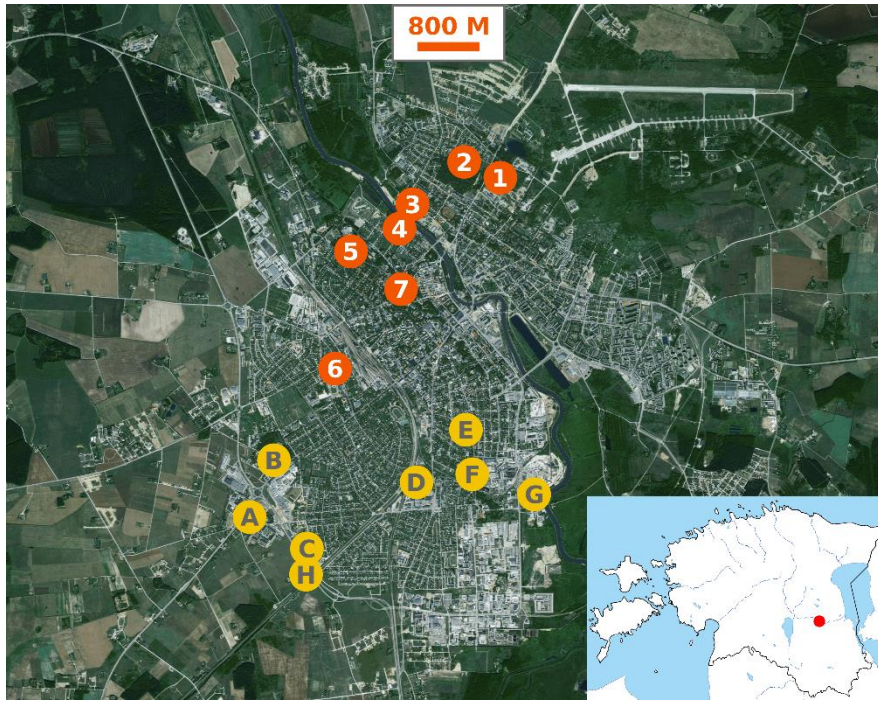

Figure 2: Map showing the collection sites in Tartu as well as the location of the town in Estonia. Sites 1, 2, 3, 4, 5 and 6 were used in 2013, 2016 and 2017. Site 7 was included in the study in 2016 and 2017. Sites A, B, C, D, E, F, G and H were added in 2017. Base map of Tartu: Estonian Land Board, 2019. Map of Estonia: ©https://d-maps.com, 2007-2021. Adapted from **II**.

4.2. Specimen collection and storage

Adult mosquitoes were collected from May to October from 2008 to 2020 (**I**, **II**, **III**), using various trapping methods. Battery-operated Mosquito Magnet Independence traps (Woodstream Corp., Lancaster, USA) baited with 1-Octen-3-ol ($C_8H_{16}O$) were used most frequently (**I**, **III**), but mosquitoes were also collected with handheld 50 cm diameter sweep nets (**I**, **II**, **III**) as well as EVS light traps (BioQuip Products, Rancho Dominguez, USA) baited with dry ice (**III**), Malaise traps (cf. Tomasson *et al.* 2014) (**III**) and window traps (cf. Sammet *et al.* 2016) (**III**). Baited traps like the Mosquito Magnet and EVS light trap specifically attract blood seeking insects, collecting primarily female mosquitoes, blackflies (*Simuliidae*), biting midges (*Ceratopogonidae*), etc. However, mosquitoes that feed on humans and other mammals are more likely to be enticed by these traps compared to, for example, bird biting species (Lühken *et al.* 2014; Sant'Ana *et al.* 2014). Baited traps were emptied

every 2 to 4 days, while Malaise and window traps were inspected a few times from spring to fall. During the study of urban mosquitoes, specimens were collected weekly from May to October (**II**) (Fig. 2). The collection protocol consisted of making two times 25 swings with a 50 cm diameter sweep net and gathering the caught specimens with an aspirator. Fieldwork in the town of Tartu was started at five o'clock in the afternoon and the sites were visited in a varying order. Sweep net catches were likely influenced by how attracted mosquitoes are to different people (Ellwanger *et al.* 2021). Finally, mosquitoes were stored as either dry material in -20 °C freezers (**I**, **II**, **III**) or in 75% ethanol (C_2H_5OH) at +4 °C or at room temperature (**II**, **III**).

4.3. Morphological identification and illustrative photos

Mosquitoes were identified under a stereomicroscope Olympus SZ61 (Olympus Corporation, Tokyo, Japan) to species or species group level, based on their morphological characteristics, using standard identification keys (**I**, **II**, **III**) (Cranston *et al.* 1987; Snow 1990; Becker *et al.* 2010). More specifically, specimens were determined to group level when identifying mosquitoes belonging to the *Aedes annulipes* group, *Anopheles maculipennis* complex and *Culex pipiens* Linnaeus/ *Cx. torrentium* Martini species (**II**, **III**). Insects kept as dry material at -20 °C were placed on ice packs for the duration of the identification process to avoid rapid melting. Individuals too damaged for identification were not included in the study. However, specimens in good physical condition were used for illustrative photos. These pictures were taken using the Leica stereo microscope M205-C (Leica Microsystems, Wetzlar, Germany) and were arranged into plates in Adobe Photoshop CS5 Extended (Adobe, San Jose, USA). Image quality was improved using Sharpen AI (Topaz Labs, Addison, USA).

4.4. Genetic identification

4.4.1. DNA extraction and PCR

Genetic methods were used to verify the results of morphological identification (**III**) and to investigate DNA variability within species (**I**). Either whole insects or up to three legs from individual specimens were used for the analyses (**I, III**). DNA extraction was conducted using

either the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) in accordance with manufacturer's instructions (**I**, **III**) or the PrepMan Ultra Sample Preparation Reagent (Thermo Fisher Scientific Inc., Waltham, USA) based on a previously published protocol (**I**) (Lilja *et al.* 2018). Finally, DNA samples were stored at -20 °C.

Genetic mosquito identification was done based on the 710 bp partial cytochrome c oxidase subunit 1 (COI), 450 bp NADH dehydrogenase subunit 5 (ND5) as well as the 368 to 387 bp ribosomal internal transcribed spacer 2 (ITS2) sequences, while the 600 bp *Wolbachia* surface protein (wsp) gene sequence (**I**, **III**) was used for detecting the intercellular symbiont *Wolbachia pipientis* (Table 1). In all cases, the polymerase chain reaction (PCR) mixes included 1 µl of template DNA, 12.5 µl of DreamTaq DNA Polymerase Master Mix (2X) (Thermo Fisher Scientific Inc. Waltham, USA), 0.5 µl of 20 µM forward and reverse primers (TAG Copenhagen, Frederiksberg, Denmark) as well as 10.5 µl of nuclease-free water (**I**, **III**). As needed, 1.0 µl of 25 mM $MgCl_2$ (Thermo Fisher Scientific, Waltham, USA) or 0.5 µl of dimethyl sulfoxide (DMSO) (ITW Reagents Division, Glenview, USA) were added to the reaction mix at the expense of nuclease-free water (**III**).

Table 1: Forward and reverse primer sequences used in this thesis.

Marker	Primer name	Sequence	Reference
COI	LCO1490	5'- GGT CAA CAA ATC ATA AAG ATA TTG G-3'	(Folmer *et al.* 1994)
	HCO2198	5'- TAA ACT TCA GGG TGA CCA AAA AAT CA -3'	
ND5	6500	5'- TCC TTA GAA TAA AAT CCC GC -3'	(Birungi & Munstermann 2002)
	7398	5'- GTT TCT GCT TTA GTT CAT TCT TC -3'	
ITS2	5.8S	5'- TGT GAA CTG CAG GAC ACA TG -3'	(Collins & Paskewitz 1996)
	28S	5'- ATG CTT AAA TTT AGG GGG TA -3'	
wsp	wsp 81F	5'- TGG TCC AAT AAG TGA TGA AGA AAC -3'	(Braig *et al.* 1998)
	wsp 691R	5'- AAA AAT TAA ACG CTA CTC CA -3'	

In the case of paper **I**, the PCR program for amplifying COI consisted of an initial denaturation at 95 °C for 2 min 15 sec, followed by 35 cycles of 95 °C for 30 sec, 57 °C for 45 sec, 72 °C for 45 sec and a final elongation step at 72 °C for 5 min. However, paper **III** included some mosquito specimens with which successful DNA replication was more difficult, therefore the PCR program for COI comprised of a denaturation stage at 94 °C for 15 min, followed by 60 cycles of 94 °C for 30 sec, 44 °C for 30 sec and 72 °C for another 30 sec, finally capped by a 10 min syntheses stage at 72 °C. On the other hand, paper **I** also required the amplification of ITS2 and ND5 sequences. The PCR program for ITS2 consisted of denaturation at 95 °C for 2 min 15 sec, then 35 cycles of 95 °C for 30 sec, 45 °C for 45 sec, 72 °C for 45 sec and a final elongation step at 72 °C for 5 min. The program for ND5 sequences involved denaturation at 94 °C for 3 min, followed by 60 cycles of 94 °C for 30 sec, 38 °C for 30 sec, 65 °C for 45 sec and a last elongation at 65 °C for 3 min. However, *Wolbachia* symbionts wsp gene sequences were amplified using a previously published protocol (Shaikevich *et al.* 2019b).

4.4.2. Electrophoresis and sequencing

The success of DNA amplification was evaluated using electrophoresis (**I**, **III**). For this purpose, 6 µl of each PCR sample was mixed with 1µl DNA Gel Loading Dye (6X) (Thermo Fisher Scientific Inc., Waltham, USA) and pipetted on to a 1.6% agarose gel infused with ethidium bromide ($C_{21}H_{20}BrN_3$). GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific Inc., Waltham, USA) or GeneRuler 100 bp Plus DNA Ladder (same company) were used to confirm that the correct marker regions had been amplified during PCR. Electrophoresis was run for up to 1 h at 120 V and 70 mA. PCR products displaying the appropriate positive signals were delivered to the Institute of Genomics Core Facility (University of Tartu, Tartu, Estonia), where the samples were cleaned and sequenced with Applied Biosystems 3130xl Genetic Analyzer, using a two-directional procedure (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA).

4.4.3. Sequence assembly and analysis

The results of sequencing were checked for quality, forward and reverse strands of each mosquito were aligned and assembled into consensus sequences as well as trimmed in BioEdit version 7.2.6.1

(**I**, **III**) (Hall 1999). For simple species identification (**III**), sequences were compared to publicly available information using the US National Library of Medicine nucleotide BLAST tool (National Institutes of Health, Bethesda, USA) and Barcode of Life Data (BOLD) Systems identification engine (Ratnasingham & Hebert 2007). More in-depth analyses were carried out with the free software MEGAX version 10.0.5 (**I**) (Kumar *et al.* 2018). For paper **I**, genetic information from 54 Estonian mosquitoes, two COI sequences from Sweden, one shared by the Swedish National Veterinary Institute (SVA) and the other by authors of a previous article (Tingström *et al.* 2016), as well as 22 sequences downloaded from GenBank (National Institutes of Health, Bethesda, USA) were used for further study. Species included in this work were *Ae. abserratus* (Felt & Young), *Ae. cataphylla* Dyar, *Ae. churchillensis*, *Ae. communis*, *Ae. hexodontus* Dyar, *Ae. punctor* (Kirby), *Ae. tahoensis* as well as *An. messeae* Falleroni, which was used as an outgroup. COI, ND5 and ITS2 regions were analyzed separately, sequences were aligned with the Multiple Sequence Comparison by Log-Expectation (MUSCEL) tool. The most appropriate models for phylogenetic analyses were chosen based on the results of the Find Best-Fit Substitution Model function. Phylogenetic trees were constructed using the Maximum Likelihood method, with bootstrap replications set to 1 000.

4.5. Statistical analysis

For paper **II**, each mosquito collection event in Tartu was supplemented with weather information recorded by the Tartu-Tõravere station of the Estonian Weather Service. This included temperature (°C), relative humidity (%), wind speed (m/s) and atmospheric pressure at sea level (hPa). Furthermore, the effective number of species (ENS) was chosen as the measure of species diversity within a single catch and calculated using the equation:

$$ENS = \exp(-\sum_{i=1}^{S} p_i \ln p_i),$$

where S is the total number of species and p is the number of specimens of the same species divided by the number of all individuals. On further inspection of the collection results, it became evident that the mosquito count data followed a Poisson distribution. For that reason, the average

number of specimens was represented with lambda (λ) and 95% confidence intervals (CI) had to be used to characterize dispersion.

Statistical analyses were carried out using R version 3.6.1 (**II**) (R Core Team 2019). The dataset was cleaned of incomplete fieldwork days, when heavy rain prevented the sampling of all sites. Independent variables were checked for mutual correlation using the appropriate function of the R package "psych" (Revelle 2020) and removed if necessary. After these considerations, the independent variables left in the study were sampling site, month and year as well as temperature, wind speed and mosquito gender. Next, generalized linear models (GLMs) were used to analyze how independent variables affected the number of mosquitoes and the ENS at sampling sites. Negative binomial regression (Venables & Ripley 2002) was used in the first case and Poisson regression in the second. Non-significant variables were removed manually. R packages "DHARMa" (Hartig 2020), "performance" (Lüdecke *et al.* 2021) and "mctest" (Imdad *et al.* 2016; Imdad & Aslam 2018) were employed to test for over- and under dispersion, zero inflation as well as multicollinearity, respectively. Package "ggplot2" (Wickham 2016) was used to create figures. The strength of the associations between variables were calculated using non-parametric Kendall rank correlation coefficient.

4.6. Data storage and accessibility

The original data generated by this research has been made publicly available. Relevant DNA sequences were uploaded to GenBank at https://www.ncbi.nlm.nih.gov/genbank/ (**I**, **III**). Tables containing mosquito count data have been stored at Figshare (Digital Science, London, UK) at https://figshare.com/ (**II, III**). Voucher specimens for all of the mosquito species collected from 2008 to 2020 in Estonia were deposited to the Entomological collection [IZBE] of the Estonian University of Life Sciences (**III**).

4.7. Technical note on nomenclature

In paper **I**, *Ochlerotatus* Reinert is treated as a genus based on the work of John F. Reinert (2000), which was later elaborated on in additional publications (Reinert *et al.* 2004, 2009). Hence, in paper **I** *Aedes communis* is written as *Ochlerotatus communis*, *Aedes punctor* as *Ochlerotatus punctor*, etc. However, *Ochlerotatus* is once again treated as a subgenus in papers **II**

and **III**, as was suggested by Wilkerson *et al.* (2015). Both competing taxonomic classifications are currently used (Robert *et al.* 2019; Culverwell *et al.* 2021). In the interest of cohesiveness, *Ochlerotatus* is used as a subgenus throughout this thesis. This has no influence on the results of paper **I**.

5. RESULTS

5.1. Genetic variability among Nordic-Baltic *Aedes communis* (De Geer) (I)

12 Estonian mosquitoes, identified as *Ae. communis* based on morphological characteristics, appeared to be more closely related to North-American sister species than *Ae. communis*, according to mitochondrial COI and ND5 sequences. Moreover, two COI sequences received from colleagues in Sweden also clustered together with the aberrant Estonian *Ae. communis*, hereafter called *Ae. communis* novel mitochondrial lineage (*Ae. communis* NML). On the other hand, the *Ae. communis* NML specimens grouped together with all other *Ae. communis* when the ITS2 marker region was analyzed. None of the examined *Ae. communis* had a positive signal for the wsp gene of the *Wolbachia* symbiont, which could have offered an explanation for the intraspecific variation within mitochondrial DNA. Additionally, sequences from *Ae. punctor*, *Ae. hexodontus*, *Ae. cataphylla* and *An. messeae*, were used to provide further context to the interspecific relationships surrounding *Ae. communis*.

COI sequences made up the majority of available reference material relating to the *Aedes communis* complex in GenBank. This publicly available data made it possible to compare the Estonian and Swedish *Ae. communis* NML to sequences from sister species *Ae. tahoensis* and *Ae. churchillensis* (Fig. 3). The between groups genetic distance between the normal *Ae. communis* sequences and those of the deviant specimens is 0.063 (Standard error (from here onwards given as ±S.E.) ±0.018) substitutions per base. At the same time, the *Ae. communis* NML cluster is genetically closer to both *Ae. tahoensis* and *Ae. churchillensis*, with a difference of 0.046 (±0.013) and 0.054 (±0.015) substitutions per base, respectively. It is noteworthy that within group distances of the *Ae. communis* NML cluster are less than 0.001 (±0.001), while the same parameter is 0.007 (±0.03) in the case of the traditional *Ae. communis* COI sequences.

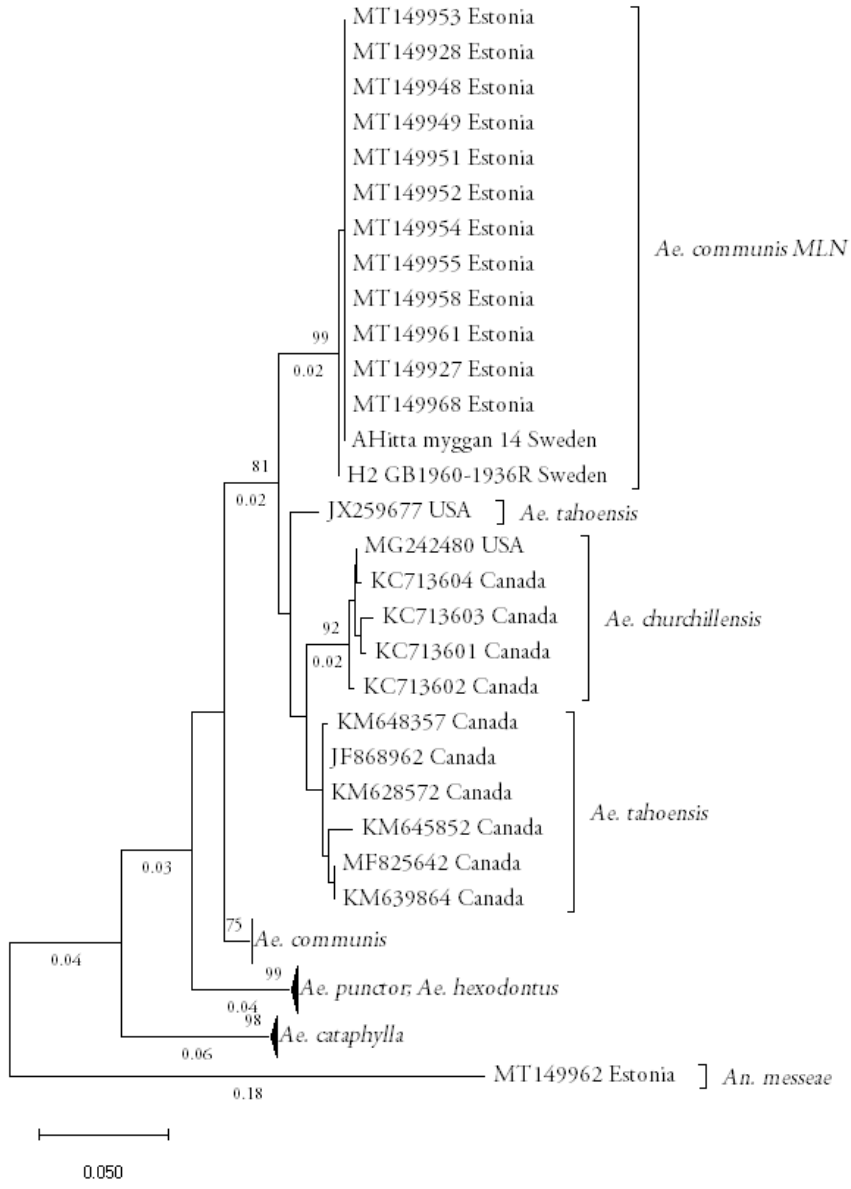

Figure 3: Phylogenetic tree based on partial COI sequences, showing the difference between traditional *Aedes communis* COI sequences and those of *Ae. communis* NML. The tree is constructed based on the Maximum Likelihood method, using Tamura 3-parameter model (Tamura 1992), with discrete gamma distribution (6 categories (+G, parameter = 0.1790)) and inferred from 72 COI sequences. The final data set contained 441 positions. Branch lengths are shown to scale and calculated based on the number of substitutions per site. Figure adapted from **I**.

Regrettably, *Ae. communis* was the only *Aedes communis* complex species with ND5 sequences available in GenBank, thus limiting the comparison with other closely related taxa. However, the genetic difference between traditional *Ae. communis* and the deviant sequences is still apparent when looking at the ND5 marker region (Fig. 4). In this case, the *Ae. communis* NML cluster is separated from other *Ae. communis* by 0.083 (±0.031) nucleotide substitutions per site. This is comparable to the differences between the other species used in the analysis. On the other hand, within group distances of the *Ae. communis* and the *Ae. communis* NML clusters remain relatively small, being 0.008 (±0.009) and 0.001 (±0.001), respectively. In fact, the *Ae. communis* NML sequences exhibit the lowest genetic variability of all analyzed groups.

Genetic discrepancies among the different *Ae. communis* specimens disappear when examining the ribosomal ITS2 sequences (Fig. 5). More specifically, the within group variation of the whole *Ae. communis* cluster is less than 0.001 (±0.001) substitutions per base. Although, this does not appear completely unusual, as the two *Ae. churchillensis* sequences downloaded from GenBank proved to be similarly identical to each other. On the other hand, there is a distance of by 0.025 (±0.009) substitutions per base between *Ae. communis* and *Ae. churchillensis*.

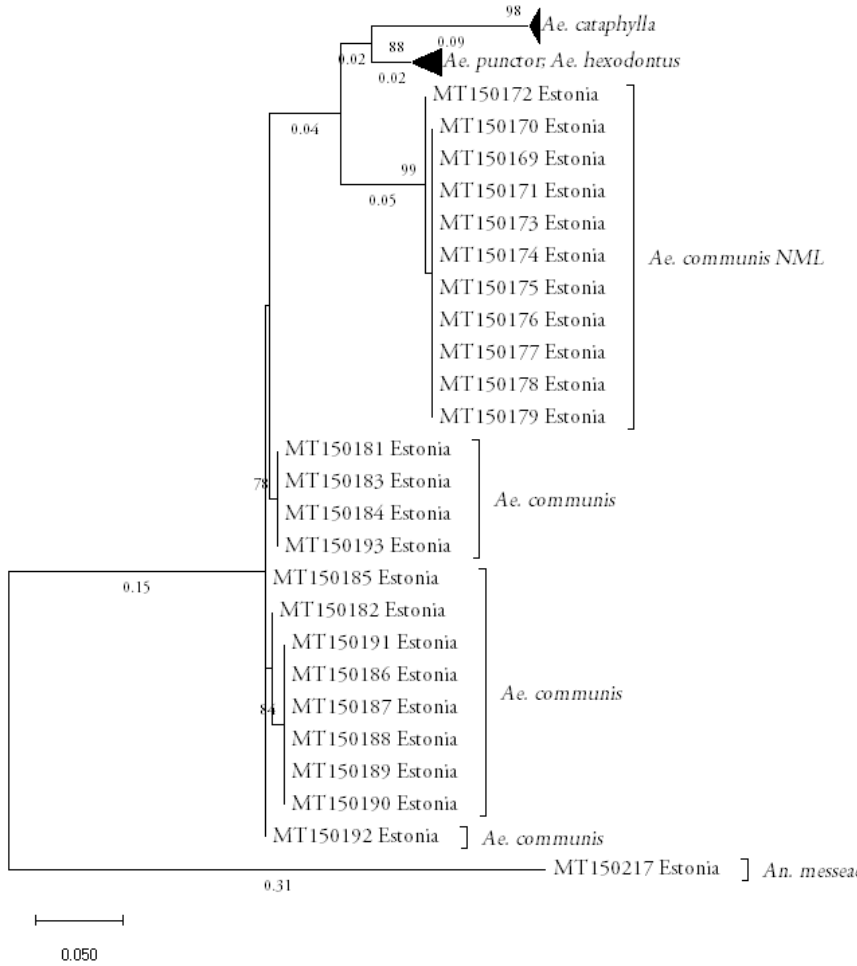

Figure 4: Phylogenetic tree based on ND5 marker region, illustrating the difference between traditional *Aedes communis* COI sequences and those of *Ae. communis* NML. The tree is calculated with the Maximum Likelihood method and using the Tamura 3-parameter model (Tamura 1992), with a discrete gamma distribution (6 categories (+G, parameter = 0.1516)). Analysis involved 48 ND5 marker sequences, with 321 positions in the final data set. Branch lengths are shown to scale and based on the number of substitutions per site. Figure adapted from **I**.

Figure 5: Phylogenetic tree based on the ITS2 marker, showing no genetic differences between the sequences of *Aedes communis*. The tree is calculated with the Maximum Likelihood method, using the Kimura 2-parameter model (Kimura 1980) and discrete Gamma distribution (6 categories (+G, parameter = 0.8737)). This is based on 53 ITS2 sequences containing 251 positions. Figure adapted from **I**.

5.2. Weather-dependent changes in the urban mosquito fauna (II)

5.2.1. Fluctuations in mosquito abundance

In total, 1 890 mosquitoes were collected from Tartu using a sweep net during three study years. Of these, 47 specimens were too damaged for identification and were excluded from the study. Interestingly, the mean number of collected mosquitoes per month decreased from 6.41, 95% CI [6.22 – 6.61] (Poisson lambda (λ), 95% CI [lower limit – upper limit]) in 2013 to 2.53, 95% CI [2.41 – 2.65] in 2017 (Fig. 6). The number of collected mosquitoes was influenced by year, month, temperature, wind conditions, insect gender and study site as well as by the associations between these factors (Table 2).

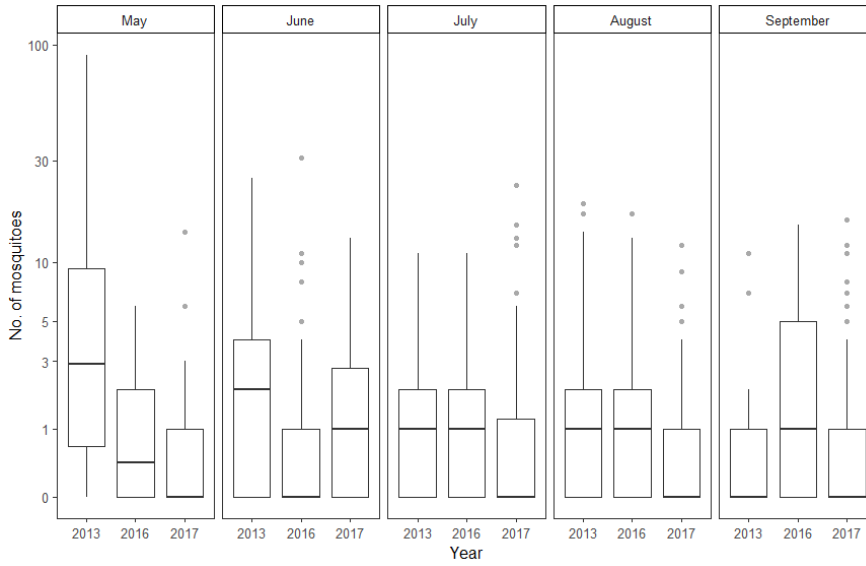

Figure 6: Average number of mosquitoes collected during the study period, grouped by month as well as year. Boxplots showing the median (dark line dividing the box), interquartile range (IQR) containing 50% of the data points (length of the box), lower (Q1) and upper (Q3) quartiles (whiskers) and outliers (gray dots). Outliers are data points with values higher than the upper fence (Q3+1.5*IQR). Y-axis is shown in the logarithmic scale. Figure adapted from **II**.

Table 2: Generalized linear model results showing how independent variables influenced the mean number of collected mosquitoes in Tartu, Estonia. The table is displaying estimates (β), confidence limits and significance (p value). Significance symbols: 0.05 to 0.01 = "*", 0.01 to 0.001 = "**", < 0.001 = "***". For further details see Table 1 in **II**.

Variable	**β**	**CI 2.5%**	**CI 97.5%**	**p value**	
(Intercept)	3,526	2,40	4,63	<0.001	***
Study site (ref: site A)					
Site B	0,852	-0,077	1,817	0,077	
Site C	1,080	0,168	2,032	0,024	*
Site D	1,465	0,601	2,380	0,001	**
Site E	2,092	1,244	2,996	<0.001	***
Site F	2,789	1,957	3,680	<0.001	***
Site G	1,637	0,773	2,553	<0.001	***
Site H	2,762	1,928	3,654	<0.001	***
Site 1	1,440	0,663	2,272	<0.001	***
Site 2	0,070	-0,739	0,928	0,871	
Site 3	0,197	-0,609	1,053	0,645	
Site 4	0,731	-0,064	1,578	0,082	
Site 5	0,534	-0,267	1,386	0,207	
Site 6	1,231	0,446	2,070	0,003	**
Site 7	0,806	-0,003	1,666	0,061	
Collection year (ref: 2013)					
2016	-1,948	-2,617	-1,287	<0.001	***
2017	-3,627	-4,315	-2,962	<0.001	***
Collection month (ref: May)					
June	-0,479	-1,117	0,159	0,175	
July	-1,844	-2,492	-1,206	<0.001	***
August	-1,584	-2,234	-0,943	<0.001	***
September	-3,558	-4,366	-2,764	<0.001	***
Gender (ref: Female)					
Male	-0,887	-1,435	-0,335	<0.001	***
Temperature	-0,099	-0,138	-0,060	<0.001	***
Wind conditions	-0,129	-0,262	0,005	0,047	*

Table 2 (Contd.)

Variable	β	CI 2.5%	CI 97.5%	p value	
Interactions between year (ref: 2013) and month (ref: May)					
2016: June	0,675	-0,217	1,564	0,125	
2017: June	1,639	0,823	2,458	<0.001	***
2016: July	2,167	1,285	3,052	<0.001	***
2017: July	2,743	1,909	3,586	<0.001	***
2016: August	1,601	0,750	2,453	<0.001	***
2017: August	1,673	0,861	2,491	<0.001	***
2016: September	3,390	2,457	4,329	<0.001	***
2017: September	4,026	3,104	4,959	<0.001	***
Interactions between month (ref: May) and insect gender (ref: Female)					
June: Male gender	-0,175	-0,872	0,518	0,607	
July: Male gender	0,229	-0,452	0,906	0,493	
August: Male gender	0,817	0,135	1,496	0,014	*
September: Male gender	1,197	0,507	1,884	<0.001	***

On average, more mosquitoes were caught at the beginning of the season, specifically in May and June, than during the rest of the fieldwork months. Mosquitoes were especially numerous in May of 2013. Surprisingly, higher temperatures appeared to correlate with fewer collected specimens on average (Fig. 3 in **II**). However, there was also a significant negative association between temperature and relative humidity (Fig. 4 in **II**), which may mean that the decrease in mosquito abundance was in reality due to the dry conditions. More predictably, the mean value of collected specimens was also lower during higher wind speeds. Also, mosquitoes were significantly more abundant at some study sites than others.

5.2.2. Changes in species diversity

Mosquitoes collected in Tartu belonged to five genera: *Aedes* Meigen, *Anopheles* Meigen, *Coquillettidia* Dyar, *Culex* Linnaeus and *Culiseta* Felt (Fig. 7). The total number of different species varied little throughout the three years, as 14 species and species groups were caught in 2013, 16 in 2016 and 17 in 2017 (Table 3 in **II**). The late summer mosquitoes *Cx. pipiens*/ torrentium were abundant every year, together resulting in 516 collected individuals (27.3% of total) over the study period. However, the abundance of other collected species varied.

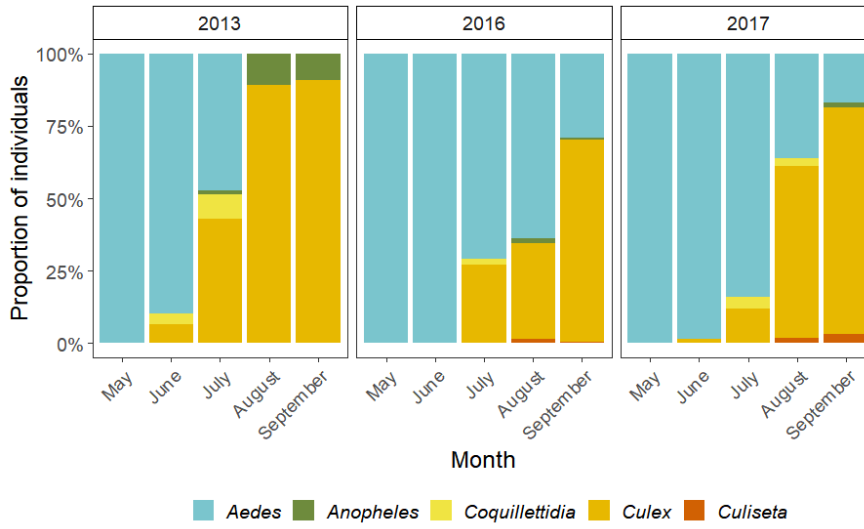

Figure 7: Figure showing the relative abundance of the different mosquito genera throughout the study years. Figure adapted from **II**.

The average effective number of species (ENS) decreased from 1.59, 95% CI [1.40 – 1.78] in 2013 to 1.11 [0.99 – 1.23] in 2017. Species diversity was influenced by both the collection year and site (Table 3). More specifically, compared to 2013, the average ENS was significantly lower in 2017, although not in 2016. Furthermore, five collection sites had on average a higher ENS compared to the reference site A.

Table 3: Generalized linear model results showing the independent variables which influenced the effective number of species (ENS) of urban mosquito fauna in Tartu, Estonia. The table is displaying estimates (β), confidence limits and significance (p value). Significance symbols: 0.05 to 0.01 = "*", 0.01 to 0.001 = "**", < 0.001 = "***". For further details see Table 2 in **II**.

Variable	β	CI 2.5%	CI 97.5%	p value	
(Intercept)	-0,065	-0,835	0,58	0,856	
Collection sites (ref: site A)					
Site B	0,442	-0,382	1,318	0,301	
Site C	0,747	-0,019	1,589	0,065	
Site D	0,747	-0,019	1,589	0,065	
Site E	1,269	0,571	2,069	< 0.001	***
Site F	1,541	0,868	2,326	< 0.001	***
Site G	0,636	-0,149	1,489	0,123	
Site H	1,598	0,925	2,383	< 0.001	***
Site 1	0,893	0,243	1,665	0,013	*
Site 2	0,343	-0,330	1,130	0,352	
Site 3	0,180	-0,502	0,974	0,628	
Site 4	0,553	-0,110	1,333	0,129	
Site 5	0,256	-0,422	1,046	0,490	
Site 6	0,737	0,082	1,513	0,041	*
Site 7	0,328	-0,374	1,134	0,388	
Study years (ref: 2013)					
2016	-0,109	-0,319	0,102	0,311	
2017	-0,628	-0,881	-0,381	< 0.001	***

5.3. Updated Estonian mosquito checklist (III)

The updated Estonian mosquito checklist came to 34 species, compared to the 30 species reported on the historic list (Remm 1957) (Table 4). Of these 34 species, 27 were confirmed with recent finds. In total, 24 344 (94.2% female) adult mosquitoes were collected from 2008 to 2020, including specimens belonging to the genera *Aedes*, *Anopheles*, *Coquillettidia*, *Culex* and *Culiseta*. Regrettably, the mosquitoes collected by H. Remm during the first half of the last century had not been preserved and therefore could not be re-examined. The species *Cs. bergrothi* (Edwards) was included in the checklist based on reports of an Estonian specimen stored at the Zoological Institute of the Russian Academy of Sciences (Khalin & Aibulatov 2020). Maps indicating historic as well as new collection points were constructed for each species (Fig. 2 to Fig. 5 in **III**). Photos of the collected mosquito species have been provided for illustrative purposes (Fig. 8 to Fig. 12). When it comes to closely related sister species that cannot be distinguished based on morphological characteristics, only the pictures of one taxon are shown.

Table 4: Comparison of the mosquito species reported in the first Estonian checklist by H. Remm (1957), species collected during the fieldwork from 2008 to 2020 and taxa included in the final updated list.

Species	Remm 1957	Collected from 2008 to 2020	Updated checklist	Figure
Aedes (*Aedes*) *cinereus* Meigen, 1818	+	+	+	Fig. 8: A, B
Aedes (*Aedimorphus*) *vexans* (Meigen, 1830)	+	+	+	Fig. 8: C, D
Aedes (*Ochlerotatus*) *annulipes* (Meigen, 1830)	+	+	+	Fig. 8: E, F
Aedes (*Ochlerotatus*) *cantans* (Meigen, 1818)	+	+	+	Fig. 8: G, H
Aedes (*Ochlerotatus*) *caspius* (Pallas, 1771)	+	+	+	Fig. 8: I, J
Aedes (*Ochlerotatus*) *cataphylla* Dyar, 1916	+	+	+	Fig. 8: K, L

Table 4 (Contd.)

Species	Remm 1957	Collected from 2008 to 2020	Updated checklist	Figure
Aedes (*Ochlerotatus*) *communis* (De Geer, 1776)	+	+	+	Fig. 9: A, B
Aedes (*Ochlerotatus*) *cyprius* Ludlow, 1920	+	+	+	Fig. 9: G, H
Aedes (Ochlerotatus) diantaeus Howard, Dyar & Knab, 1913	+	+	+	Fig. 9: I
Aedes (Ochlerotatus) dorsalis (Meigen, 1830)	+	-	+	
Aedes (*Ochlerotatus*) *excrucians* (Walker, 1856)	+	+	+	Fig. 9: C, D
Aedes (*Ochlerotatus*) *flavescens* (Müller, 1764)	+	+	+	Fig. 9: E, F
Aedes (*Ochlerotatus*) *hexodontus* Dyar, 1916	-	+	+	Fig. 10: E, F
Aedes (*Ochlerotatus*) *intrudens* Dyar, 1919	+	+	+	Fig. 10: G, H
Aedes (*Ochlerotatus*) *leucomelas* (Meigen, 1804)	+	+	+	Fig. 10: I, J
Aedes (*Ochlerotatus*) *nigrinus* (Eckstein, 1918)	+	-	+	
Aedes (*Ochlerotatus*) *punctor* (Kirby, 1837)	+	+	+	Fig. 10: A, B

Table 4 (Contd.)

Species	Remm 1957	Collected from 2008 to 2020	Updated checklist	Figure
Aedes (*Ochlerotatus*) *sticticus* (Meigen, 1838)	-	+	+	Fig. 10: C, D
Aedes (Ochlerotatus) riparius Dyar & Knab, 1907	+	-	+	
Anopheles (*Anopheles*) *algeriensis* Theobald, 1903	+	-	+	
Anopheles (*Anopheles*) *claviger* (Meigen, 1804)	+	+	+	Fig. 11: E, F
Anopheles (*Anopheles*) *maculipennis s.s.* Meigen, 1818	+	+	+	
Anopheles (*Anopheles*) *messeae* Falleroni, 1926	-	+	+	Fig. 11: G, H
Anopheles (*Anopheles*) *plumbeus* Stephens, 1828	+	-	+	
Coquillettidia (*Coquillettidia*) *richiardii* (Ficalbi, 1889)	+	+	+	Fig. 11: I, J
Culex (*Culex*) *pipiens* Linnaeus, 1758	+	+	+	
Culex (*Culex*) *torrentium* Martini, 1925	+	+	+	Fig. 11: A, B
Culex (*Neoculex*) *territans* Walker, 1856	+	+	+	Fig. 11: C, D

Table 4 (Contd.)

Species	Remm 1957	Collected from 2008 to 2020	Updated checklist	Figure
Culiseta (*Culicella*) *morsitans* (Theobald, 1901)	+	+	+	Fig. 12: A, B
Culiseta (*Culicella*) *ochroptera* (Peus, 1935)	+	+	+	Fig. 12: E, F
Culiseta (*Culiseta*) *alaskaensis* (Ludlow, 1906)	+	+	+	Fig. 12: G, H
Culiseta (*Culiseta*) *annulata* (Schrank, 1776)	+	+	+	Fig. 12: C, D
Culiseta (*Culiseta*) *bergrothi* (Edwards, 1921)	-	-	+	
Culiseta (*Culicella*) *fumipennis* (Stephens, 1825)	+	-	+	
Total no. of species	**30**	**27**	**34**	

Aedes communis proved to be the most abundant mosquito species, with 7 316 individuals (30.1% of total) collected during fieldwork. This was followed by *Ae. punctor* and *Ae. cataphylla*, represented by 4 594 (18.9% of total) and 3 951 specimens (16.2% of total), respectively. Species that are generally active during the end of the summer were also relatively well represented: the collection effort resulted in 1 436 (5.9% of total) *Ae. cinereus* Meigen as well as 1 236 (5.1% of total) *Cx. pipiens/ torrentium*. The only *Coquillettidia* species in Estonia, *Cq. richiardii* (Ficalbi), was represented 787 mosquitoes (3.2% of total). At the same time, *An. claviger* (Meigen) proved to be the most common anopheline species in the country, with 1 038 (4.3% of total) collected individuals. In comparison, the *Anopheles maculipennis* complex was only represented by 215 specimens (0.9% of total), the majority of these being *An. messeae* according to DNA barcoding. The halophilic *Ae. caspius* (Pallas) was common on islands and near the shore of the Baltic Sea on the mainland, although only 206 individuals (0.9% of total) were collected, this was due to a relatively small number of collection sites by the sea. Interestingly, all four of the *Culiseta* species collected from 2008 to 2020 were rare, represented by less than 50 specimens. Also, only four *Ae.*

cyprius Ludlow (0.02% of total) and one *Ae. diantaeus* Howard, Dyar & Knab (0.004% of total) were identified during the study period.

Seven additional species are likely be present in Estonia based on the mosquito checklists of other Nordic-Baltic countries and may have been overlooked. These were *Ae. geminus* Peus, *Ae. geniculatus* (Olivier), *Ae. euedes* Howard, Dyar & Knab, *Ae. pullatus* (Coquillett), *Ae. punctodes* Dyar and *An. daciae* Linton, Nicolescu & Harbach.

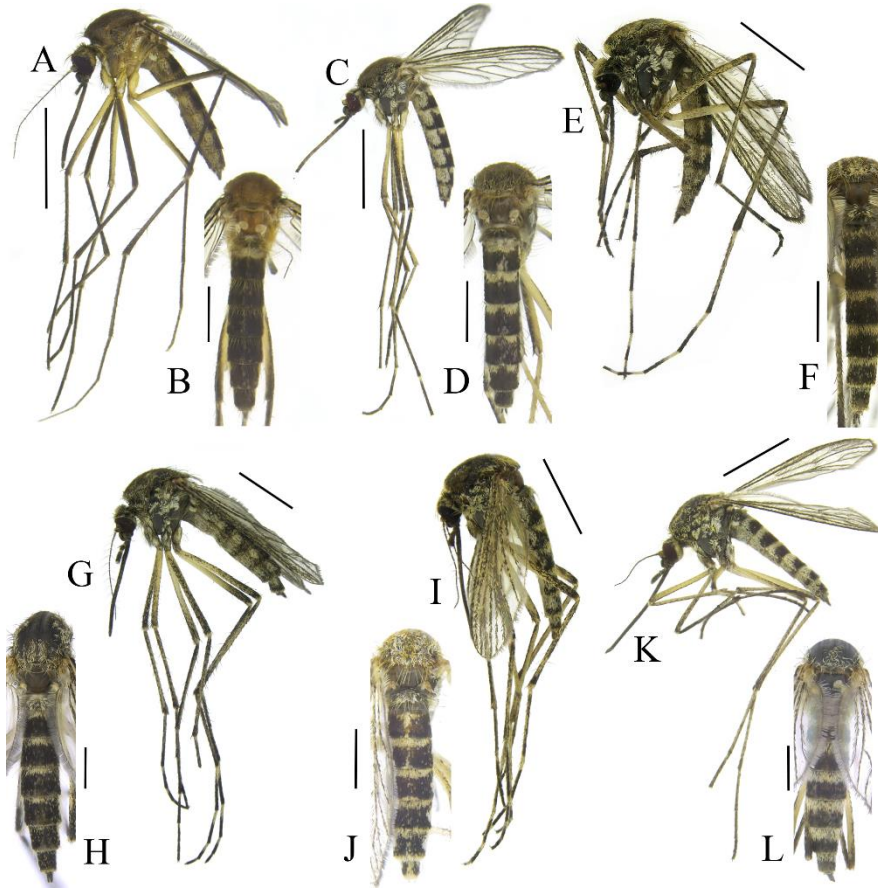

Figure 8: Habitus of female mosquitoes, lateral view (A, C, E, G, I, K), dorsal view of the abdomen (B, D, F, H, J, L). **A, B,** *Aedes (Aedes) cinereus*; **C, D,** *Aedes (Aedimorphus) vexans*; **E, F,** *Aedes (Ochlerotatus) annulipes*; **G, H,** *Aedes (Ochlerotatus) cancans*; **I, J,** *Aedes (Ochlerotatus) caspius*; **K, L,** *Aedes (Ochlerotatus) cataphylla.* Scale bar = 2 mm (A, C, E, G, I, K) and 1 mm (B, D, F, H, J, L).

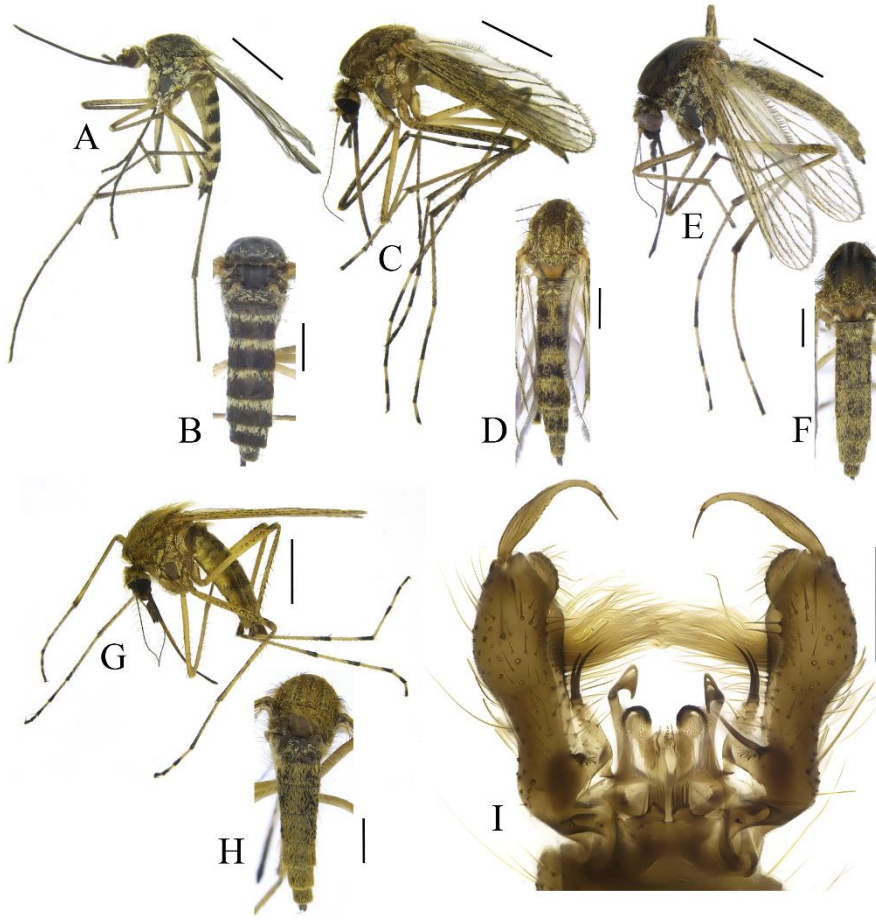

Figure 9: Habitus of female mosquitoes and a male hypopygium, lateral view (A, C, E, G), dorsal view of the abdomen (B, D, F, H), and ventral view (I). **A, B,** *Aedes* (*Ochlerotatus*) *communis*; **C, D,** *Aedes* (*Ochlerotatus*) *excrucians*; **E, F,** *Aedes* (*Ochlerotatus*) *flavescens*; **G, H,** *Aedes* (*Ochlerotatus*) *cyprius*; **I,** *Aedes* (*Ochlerotatus*) *diantaeus*. Scale bar = 2 mm (A, C, E, G), 1 mm (B, D, F, H) and 0.2 mm (I).

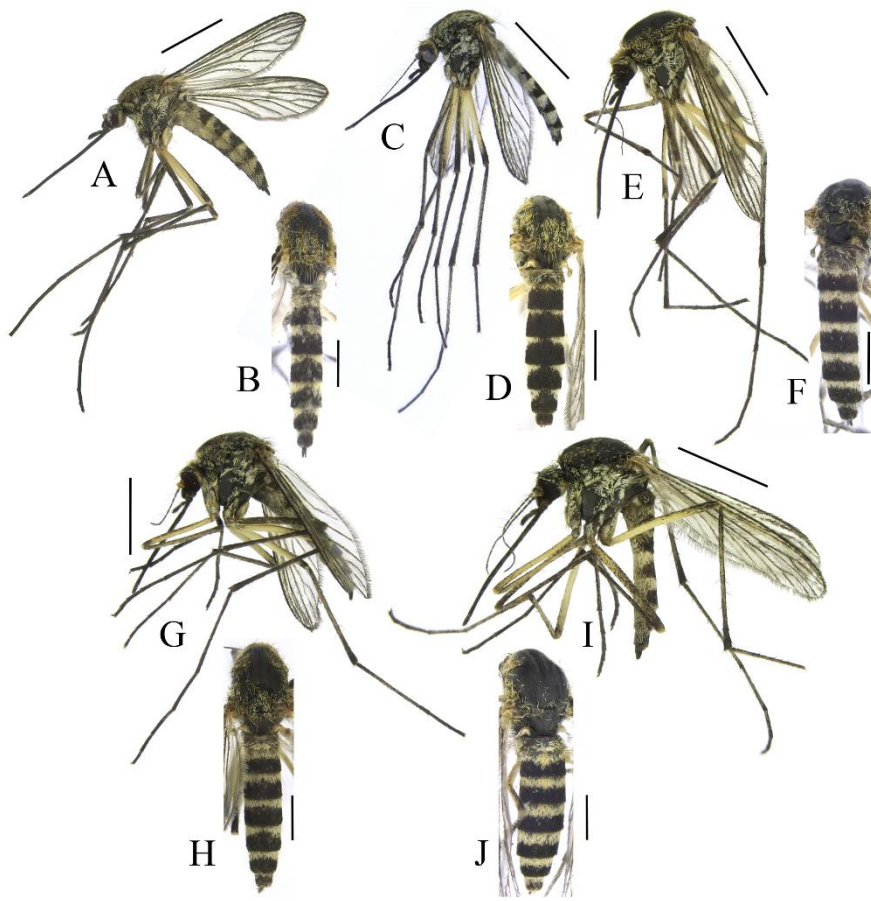

Figure 10: Habitus of female mosquitoes, lateral view (A, C, E, G, I), dorsal view of the abdomen (B, D, F, H, J). **A, B,** *Aedes (Ochlerotatus) punctor;* **C, D,** *Aedes (Ochlerotatus) sticticus*; **E, F,** *Aedes (Ochlerotatus) hexodontus*; **G, H,** *Aedes (Ochlerotatus) intrudens;* **I, J,** *Aedes (Ochlerotatus) leucomelas*. Scale bar = 2 mm (A, C, E, G, I) and 1 mm (B, D, F, H, J).

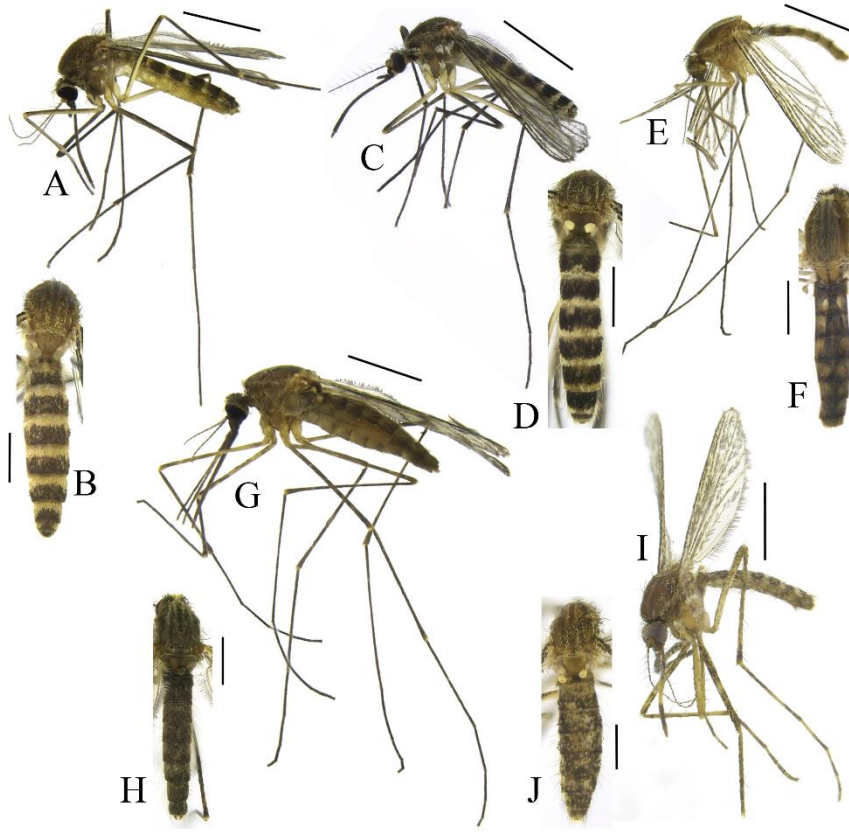

Figure 11: Habitus of female mosquitoes, lateral view (A, C, E, G, I), dorsal view of the abdomen (B, D, F, H, J). **A, B,** *Culex* (*Culex*) *torrentium*; **C, D,** *Culex* (*Neoculex*) *territans*; **E, F,** *Anopheles* (*Anopheles*) *claviger*; **G, H,** *Anopheles messeae*; **I, J,** *Coquillettidia* (*Coquillettidia*) *richiardii*. Scale bar = 2 mm (A, C, E, G, I) and 1 mm (B, D, F, H, J).

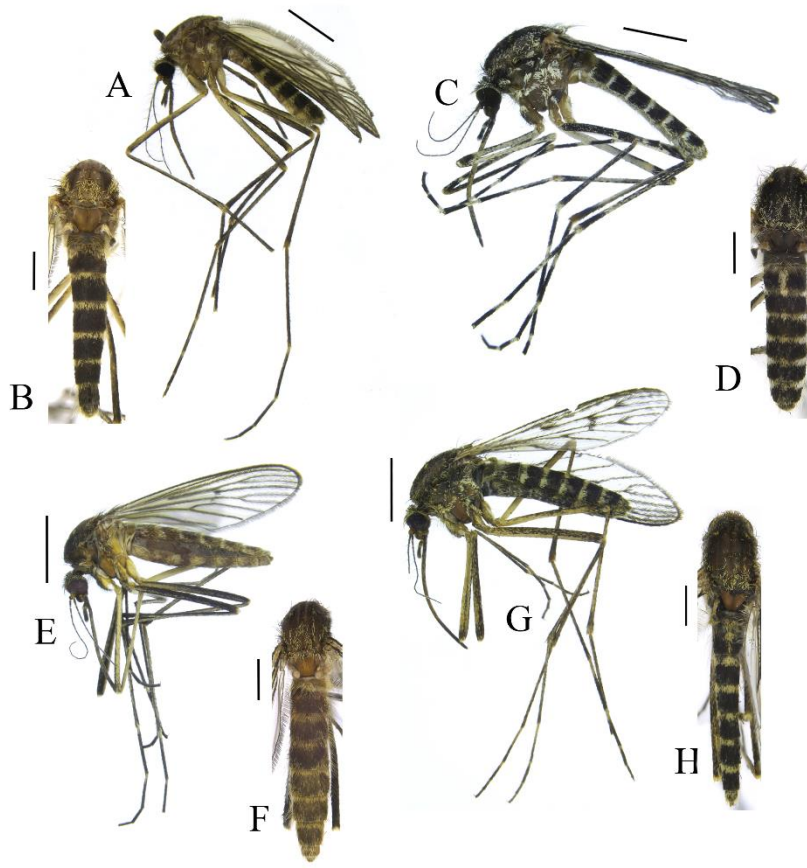

Figure 12: Habitus of female mosquitoes, lateral view (A, C, E, G), dorsal view of the abdomen (B, D, F, H). **A, B,** *Culiseta (Culicella) morsitans*; **C, D,** *Culiseta (Culiseta) annulata*; **E, F,** *Culiseta (Culicella) ochroptera*; **G, H,** *Culiseta (Culiseta) alaskaensis*. Scale bar = 2 mm (A, C, E, G) and 1 mm (B, D, F, H).

6. DISCUSSION

6.1. MtDNA lineages of *Ae. communis* and their implication for DNA barcoding (I)

Although initial DNA barcoding showed that some mosquitoes collected in Estonia and Sweden were the most closely related to the North-American species *Ae. tahoensis*, further analyses showed that this was an example of intraspecific variation within *Ae. communis*. More specifically, there appears to be two distinct mitochondrial lineages among the populations of Nordic-Baltic *Ae. communis*, while there appears to be no such division between the individuals based on the ribosomal DNA marker ITS2. The exact reason for this is unclear, but it highlights the dangers of only using mitochondrial markers for species identification.

Species with multiple distinct mtDNA lineages have been found before (Murugan *et al.* 2016) and this can result from various processes. For example, changes in mtDNA variation may be due to an infection with the *Wolbachia* bacterium, as the maternally inherited intercellular symbiont is known to produce cytoplasmic incompatibility between infected and uninfected individuals (Turelli & Hoffmann 1995). However, all of the analyzed *Ae. communis* tested negative for the *Wolbachia* surface protein, which is in agreement with previous finds (Shaikevich *et al.* 2019a; b). Therefore, there is currently no proof of the symbiont having any role in the development of the two mtDNA lineages in the Nordic-Baltic *Ae. communis*. Though, it should be added that no signs of a current infection does not rule out the possibility of some *Ae. communis* individuals being infected in the past (Yeap *et al.* 2016). On the other hand, the presence of distinct genetic lineages in the same population could also be caused by the introduction of individuals from other geographic regions, organisms of the same species adapting to different ecological niches or many other reasons (Suesdek 2019). Unfortunately, there is currently too little information to draw any real conclusions concerning the origin of the atypical mtDNA lineage found in *Ae. communis* specimens in this thesis.

It is common practice to use mitochondrial markers for the identification of species, but this can sometimes lead to inaccurate results. For example, relying solely on the often used partial COI sequence has been

found to lead to an overestimation in the number of existing species due to its relatively fast evolution rate (Frézal & Leblois 2008; Hemmerter *et al.* 2009). Furthermore, there is a chance of nuclear mitochondrial pseudogenes (NuMts) being amplified during PCR and introducing unexpected variability to the phylogenetic analysis (Song *et al.* 2008; Beebe 2018). NuMts are copies of mtDNA sequences that have been incorporated into nuclear DNA, where they become non-functional and free to accumulate nucleotide substitutions without evolutionary constraints (Lopez *et al.* 1994). NuMts can be suspected, if sequences of protein coding mitochondrial genes appear to have unusual insertions and deletions (indels) or stop codons (Buhay 2009). Another phenomenon that can influence barcoding and phylogenetic analysis is heteroplasmy: occurrence of multiple mitochondrial haplotypes in one organism (Song *et al.* 2008). This can be detected by the occurrence of more than one band when PCR products are subjected to gel electrophoresis (Frey & Frey 2004). While there are no obvious signs of NuMts or heteroplasmy when examining the *Ae. communis* NML sequences, neither can be completely ruled out at the moment. This highlights the need for careful consideration when DNA barcoding produces unexpected results as well as the necessity of using nuclear sequences in tandem with mitochondrial markers.

6.2. Weather dependent changes in the urban mosquito fauna (II)

Based on the 1890 specimens collected in 2013, 2016 and 2017 from different locations in Tartu, urban mosquito abundance varies significantly between years, months and study sites, while the species diversity appears to be more stable. In general, both the number of collected individuals as well as their diversity decreased from the first study year to the last. Even the specific makeup of the mosquito fauna proved variable, with a number of species being far more abundant during some years compared to others. This illustrates the need to include information from multiple years of fieldwork to studies of this type.

Firstly, it should be noted that springtime conditions likely had a considerable effect on the general abundance and diversity of the studied mosquitoes. Species that relay on snowmelt or flood waters are especially sensitive to circumstances in April and May (Becker *et al.* 2010). In fact, May of 2013 appears to have been especially favorable for mosquitoes,

as it was both warmer and rainier than in other years (Kallis *et al.* 2014; Loodla *et al.* 2017, 2018) and it has been previously reported, that the mean relative humidity in May can strongly influence insect abundance throughout the rest of the season (Carrieri *et al.* 2014). Additionally, in 2016 and 2017, snow cover melted numerous times during the winter as well as at the start of spring and thus, unlike in 2013, there were few flooded areas by the time mosquito larvae became active.

Temperature and wind conditions were the main weather factors influencing mosquito abundance in Tartu. There was significant negative correlation between temperature and relative humidity and only one of these variables could be included in the statistical analysis. It became apparent that higher temperatures coincided with both lower relative humidity and fewer collected mosquitoes. Coincidently, previous research has shown that urban environments generally tend to be warmer and drier than the surrounding areas (Fukui 1957; Araujo *et al.* 2015). Therefore, it is possible that as temperature rises, mosquitoes become less abundant in cities. However, this would also make for an uncomfortable environment for human beings. On the other hand, stronger winds had a predictably negative effect on the amount of collected specimens. Although moderate winds can be helpful to mosquitoes by facilitating long distance dispersal as well as carrying host scent down-wind, many species have trouble flying in windy conditions (Dufourd & Dumont 2013; Endo & Eltahir 2018).

Mosquitoes were significantly more abundant at some collections sites compared to others and a few of these locations had higher species diversity as well. It is likely that these differences were the result of local landscape factors, which were not accounted for in this study, as there did not appear to be any interactions between collection sites and weather conditions. Also, care should be taken when comparing sites A through H to sites 1 to 7 (Fig. 2), as this data was collected by different people and some of the variation could be the result of sampling bias. This is especially true as 62.6% of all collected specimens were female and were most likely differently attracted to each researcher. Although previous work has shown that many mosquito species naturally exhibit a 1:1 sex ratio and some are even male-biased (Lounibos & Escher 2008), blood-seeking females are more likely to be caught in an insect net. On the other hand, statistical analysis also revealed an interaction between mosquito gender and month. More specifically, compared to females,

male mosquitoes were collected more often in the last two months of the study period than in May. This could be in part because the females of *Cx. pipiens*, which is one of the most numerous species at the end of the summer, do not usually take a blood meal before overwintering (Becker *et al.* 2020) and thus were not especially attracted to people.

Species diversity reflected by ENS significantly decreased from 2013 to 2017. This may be because fewer mosquitoes were collected on average during the last study year and therefore less abundant species were caught more infrequently. It is unsurprising that even though the abundance of many of the collected species varied over the three years, *Cx. pipiens/ torrentium* remained relatively numerous throughout the study. This result fits well with previous research, as *Cx. pipiens* is known to do well in urban environments around the world (Dowling *et al.* 2013).

It is important to note that this study took place in only one town and more research is needed to test if these associations hold true in other urban areas situated in the same or similar climate zones. Additional studies are also needed to determine the interplay between climatic variables and the microhabitats that mosquitoes occupy in cities. It would also be beneficial to collect mosquitoes from both urban environments as well as rural areas using the same methodology, to develop a more accurate overview of the differences between the mosquito fauna of these habitats.

6.3. Rediscovering Estonian mosquitoes (III)

An updated Estonian mosquito checklist was created based on historic records and 24 344 (94.2% female) adult mosquitoes collected from 2008 to 2020, while also taking into account information from the neighboring countries. All in all, the new list includes 34 species, 27 of which were proven with newly caught voucher specimens. Unfortunately, the mosquitoes collected by H. Remm, on which the first checklist was based (Remm 1957), have not been preserved and thus their species identity could not be verified. Besides, some species are likely still missing from the checklist, requiring further collection efforts to detect.

The updated checklist came to include four new species compared to the original list, while six species with historic records were not found again during this study. Out of the new additions, the presence

of *Ae. hexodontus*, *Ae. sticticus* (Meigen) and *An. messeae* was proven by morphological as well as genetic identification. Furthermore, all of these species were represented with numerous finds. However, *Cs. bergrothi* was included on the list based on a single Estonian specimen stored at the Zoological Institute of the Russian Academy of Sciences (Khalin & Aibulatov 2020). When it comes to the species that were in the historic checklist, but were not collected again, *Ae. dorsalis* (Meigen), *Ae. nigrinus* (Eckstein) and *Ae. riparius* Dyar & Knab are most likely present in Estonia, as these species have been reported from most neighboring countries (Pakalniškis *et al.* 2000; Spungis 2000; Lundström *et al.* 2013; Culverwell 2018; Khalin & Aibulatov 2020). However, *An. plumbeus* Stephens and *Cs. fumipennis* (Stephens) appear to be much rarer in the Nordic-Baltic region: the closest collection points to Estonia are in Lithuania and Sweden for the former and Northwestern Russia as well as Sweden for the latter (Pakalniškis *et al.* 2000; Lundström *et al.* 2013; Khalin & Aibulatov 2020). Finally, *An. algeriensis* Theobald was also included on the original Estonian checklist, but the closest other country it has also been reported from is Sweden and this species is normally found in warmer areas (Scholte *et al.* 2011; Lundström *et al.* 2013).

Based on literature, seven additional species should be discussed. Firstly, *Ae. rusticus* (Rossi) has been reported as an Estonian species in many articles (Snow & Ramsdale 1999; Robert *et al.* 2019; Khalin & Aibulatov 2020), but no information regarding the collection of this species in the country can be found. It has been previously pointed out, that both *Ae. rusticus* and *Ae. cantans* (Meigen) have been historically referred to as *Ae. maculatus* Meigen (Huldén & Huldén 2014). Therefore, it is possible that the species named *Ae. maculatus* in the first Estonian checklist (Remm 1957) was later taken to mean *Ae. rusticus*, although its description as well as Remm's previous work indicate that it was actually used for *Ae. cantans*. Secondly, *Ae. geminus*, *Ae. geniculatus*, *Ae. euedes*, *Ae. pullatus*, *Ae. punctodes* and *An. daciae* could all be present in Estonia, as these species have been previously found in the Nordic-Baltic region (Pakalniškis *et al.* 2000; Spungis 2000; Lundström *et al.* 2013; Culverwell 2018; Khalin & Aibulatov 2020). *Aedes geminus*, *Ae. punctodes* as well as *An. daciae* can be especially difficult to differentiate from closely related species that are native to Estonia and could have been overlooked. Interestingly, one mosquito collected in 2017 was identified as *Ae. pullatus* based on morphological characteristics (**II**), but later DNA barcoding revealed it to be *Ae. communis* (**III**). This specimen was unusual, as it had a patch of

scales on its hypostigmal area, which is normally absent on *Ae. communis*. This further emphasizes the need to use both morphological traits as well as numerous genetic markers for species identification.

It is possible that some species were underrepresented in this dataset due to the chosen collection sites and methods. For example, much of the sampling effort was concentrated on the Estonian mainland, but salinity tolerant species like *Ae. caspius* and *Ae. dorsalis* are much more likely to be found in coastal areas. Also, the majority of specimens were collected with traps that are specifically designed to attract blood-seeking arthropods. Research has shown that some mosquitoes respond more readily to these traps than others, which can lead to underestimating the abundance of several species (Lühken *et al.* 2014; Sant'Ana *et al.* 2014). Sampling larvae and overwintering adults would give a more rounded view of the relative abundance of these mosquitoes. Furthermore, collecting additional male mosquitoes may have some benefits as well. Several species can be more readily differentiated from others based on male hypopygia than female morphology. For example, the presence of *Ae. diantaeus* in Estonia was finally verified based on a single male specimen. Therefore, future research in the country should include more diverse sampling sites and methods.

This study gives an overview of the most common mosquitoes in Estonia, creating a framework for predicting which species are more likely to contribute to the spread of vector-borne pathogens. For example, *Ae. communis* has been found to be the most abundant species in the country, which is in agreement with other research from the Nordic-Baltic area (Culverwell *et al.* 2021), and this species has been indicated as a vector of several pathogens. Importantly for Estonia, these include the tularemia bacteria *Francisella tularensis* as well as the filarial nematode *Dirofilaria repens* (Lundström *et al.* 2011; Shaikevich *et al.* 2019a). Furthermore, ornithophilic species like *Cx. pipiens* and *Cx. torrentium* are important for spreading the Sindbis virus from migrating birds to humans (Francy *et al.* 1989). Thus far, no human cases of the Sindbis fever have been reported in Estonia, but the pathogen has been detected in birds (Uryvaev *et al.* 1992) and has been responsible for outbreaks in Sweden, Finland and Russia (Kurkela *et al.* 2005; Bergqvist *et al.* 2015). It is therefore noteworthy that specimens identified as *Cx. pipiens/ torrentium* were the most numerous mosquitoes in the urban environment (**II**). On the other hand, anopheline mosquitoes capable of transmitting *Plasmodium*

vivax (Grassi & Feletti) appear to be relatively uncommon in Estonia. Therefore, the re-establishment of temperate malaria is unlikely in the country without significant changes.

Further research is needed for a more detailed overview of mosquito ecology and diversity on both global as well as local scales. As these insects have a strong effect on the wellbeing of other organisms, it is important to be able to make predictions concerning the future abundance and distribution of vector species. In Estonia, mosquitoes need to be collected more regularly and the coastal regions should be better represented in the dataset. Furthermore, there are still unanswered questions about the best morphological traits and genetic markers to use for discriminating between certain closely related species. Also, additional studies need to be conducted in the country to identify the main vectors and prevalence of pathogens like *Francisella tularensis*, *Dirofilaria repens* and the Sindbis virus. Finally, more should be learned about designing urban landscapes in a way that is comfortable for humans, but does not lead to an overabundance of blood-seeking insects.

7. CONCLUSIONS

The results of this thesis provide an updated assessment of Estonian mosquito species for the first time in over 60 years, giving an overview of their current diversity as well as abundance in both nature and urban green spaces. Considering the aims and hypothesis of the study, the main conclusions are as follows:

1. This is the first report of the common Holarctic species *Ae. communis* exhibiting two remarkably distinct mitochondrial DNA lineages in the Nordic-Baltic region (**I**).

    - DNA barcoding based on a single mtDNA marker is a valuable tool for species identification, but care should be taken when encountering ambiguous or surprising results.

2. Mosquitoes inhabiting urban green spaces in the boreal region are restricted by the dry environment, regardless of the favorability of higher temperatures, and their blood-seeking behavior is further hindered by strong winds (**II**).

3. Springtime snowmelt and the interplay between temperature and relative humidity in May have a strong effect on the whole mosquito season (**II**).

4. Even in the Nordic-Baltic region, members of the *Culex pipiens*/ torrentium group are the consistently abundant in the urban environment, while springtime species like *Ae. communis* are far more numerous in rural areas (**II, III**).

5. Estonia is home to at least 34 mosquito species based on the present estimate, but more studies are needed (**I, III**).

    - Four new species were added to the updated mosquito checklist compared to the historic data.

    - Reports of Ae. rusticus in Estonia were found to be most likely the result of a misunderstanding.

Finally, future studies should concentrate on collecting mosquitoes from Estonian islands and the coastal areas to better understand the abundance and diversity patterns of saline tolerant species. Furthermore, more work should be done to track the distribution of the vector-borne nematode *Dirofilaria repens* as well as the Sindbis virus in the country. Also, further information is required to assess the importance of mosquitoes in the transmission of the tularemia bacteria *Francisella tularensis* in the Baltic region. Furthermore, hematophagous insect surveillance protocols should be established at Tallinn Airport and the Port of Tallinn, to lessen the chance of importing invasive species. Without doubt, there is still much to learn about mosquitoes on both global and local scales.

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SUMMARY IN ESTONIAN

Pistesääsklased (Diptera: Culicidae) on laialt levinud putukate rühm, keda on praeguseks kirjeldatud 3 591 liiki (Harbach 2021). Meditsiinilisest seisukohast on neist liikidest tähtsad vaid vähesed. Nimelt vajavad mõnede pistesääsklaste emased isendid munemiseks lisavalku, mille nad omandavad selgroogsete verest. Pistesääsklaste hammustused on aga nii inimestele kui ka loomadele äärmiselt häirivad ja seeläbi võivad levida mitmesugused haigustekitajad. Seega on pistesääsklaste mitmekesisuse ja ökoloogia täpne tundmine putukate tekitatava häiringu vähendamiseks ning võimaliku nakkusohu ettenägemiseks äärmiselt tähtis.

Pistesääsklaste elutsükkel koosneb muna, vastse, nuku ja valmiku staadiumist. Seejuures saavad pistesääsklaste vastsed areneda vaid veekeskkonnas, kuigi nende täpne elupaiga nõudlus on liigiti varieeruv (Becker jt 2010). Ebasoodsate ilmastikutingimuste üleelamiseks on pistesääsklastel välja arenenud puhkestaadium ehk diapaus. Olenevalt liigist võivad diapausi jääda munad, vastsed või valmikud. See omakorda mõjutab emaste pistesääsklaste aktiivsusperioodi pikkust. Suure osa ajast toituvad nii emased kui ka isased pistesääsklased taimemahladest ja nektarist (Barredo ja DeGennaro 2020), vaid emaste pistmis-imemissuised on piisavalt tugevad, et läbistada vere imemiseks nahka.

Läbi emaste pistesääsklaste toitumiskäitumise levivad haigustekitajad põhjustavad maailmas märkimisväärset kahju. Lülijalgsete vektoritega edasi kanduvad patogeenid moodustavad ülemaailmsest haiguskoormusest umbes 17% ning ligikaudu 80% inimkonnast elab nende tõbede levikualas (WHO 2017). Seejuures on kõige sagedamad vektorid just pistesääsklased. Niisugused nakkushaigused põhjustavad nii surmasid kui ka pikaajalisi terviseprobleeme, olles koormavad nii üksikisikutele kui ka piirkonna majandusele. Ühtlasi on lähiajaloos märgatavalt muutunud nii pistesääsklaste kui ka nendega levivate haigustekitajate areaalid. Eriti just tihe rahvusvaheline kaubandus ja inimeste ning loomade reisimine on putukate ja patogeenide levikut märkimisväärselt kiirendanud (Kraemer jt 2015, Medlock jt 2012). Kohalike pistesääsklaste mitmekesisust ja arvukust mõjutavad seejuures ka mitmesugused antropogeensed protsessid, näiteks maakasutuse muutused, urbaniseerumine ja üldine sotsiaal-majanduslik areng (Brugueras jt 2020, Franklinos jt 2019). Kuigi arvatakse, et lähiajaloos aset

leidnud kliimamuutused on pistesääsklaste levikut võrreldes eelnevalt loetletud teguritega üpris vähe mõjutanud (Zell jt 2008), on klimaatilised tegurid ning nende anomaaliad putukate ja haigustekitajate jaoks siiski äärmiselt tähtsad (Brugueras jt 2020, Colón-González jt 2021, Semenza ja Suk 2018).

Eesti on Põhja- ja Baltimaades üks väheseid maid, mille kohta ei ole rohkem kui 60 viimase aasta jooksul teaduskirjanduses avaldatud uut pistesääsklaste nimekirja. Eesti esimese ja siiani ainukese niisuguse töö publitseeris professor Hans Remm 1957. aastal. Tänapäeval kasutatakse liikide tuvastamiseks peale morfoloogiliste tunnuste hindamise sageli ka DNA analüüsi. Geneetilised meetodid on bioloogilise mitmekesisuse uurimisele väga palju kaasa aidanud. Siiski tuleb eelnimetatusse suhtuda teatud ettevaatusega, sest ka laialt kasutatavate standardsete meetoditega, nagu mitokondriaalse DNA triipkoodistamine, võib kaasneda mitmesuguseid probleeme ja tulemuste väärtõlgendamist.

Parema ülevaate saamiseks Eesti pistesääsklaste liigilisest mitmekesisusest ja linnasääski mõjutavatest ilmastikuteguritest püstitati järgmised hüpoteesid.

- Laialt levinud liik harilik metsasääsk (*Aedes communis* (De Geer)) peidab endas seni uurimata krüptilist mitmekesisust.
- Linnas elavate pistesääsklaste arvukust mõjutab kõige enam temperatuur.
- Eestis on rohkem pistesääsklaste liike, kui on sedastatud prof H. Remmi pistesääsklaste nimekirjas.

Töö eesmärgid

- Anda detailsem ülevaade hariliku metsasääse geneetilisest mitmekesisusest.
- Uurida erinevate ilmastikutingimuste mõju linnas elavate pistesääsklaste arvukusele ja mitmekesisusele.
- Koostada uus Eesti pistesääsklaste kommenteeritud nimekiri.

Käesoleva töö materjal on kogutud aastatel 2008–2020 erinevatest Eesti paikadest (joonis 1) ja avaldatud kolme teadusartiklina (**I**, **II**,

III). Linnas elavaid pistesääsklasi uuriti aastatel 2013, 2016 ja 2017 (**II**, **III**), keskendudes Tartule (joonis 2). Seejuures kasutati putukate püüdmiseks erinevaid meetodeid: nii akudel töötavaid Mosquito Magnet Independence'i lõkse (**I**, **II**, **III**) (Woodstream Corp., Lancaster, USA) ja EVSi valguspüünist (**III**) (BioQuip Products, Rancho Dominguez, USA) kui ka Malaise'i püüniseid (**III**) (vt Tomasson jt 2014), akenpüüniseid (**III**) (vt Sammet jt 2016) ning putukavõrku (**I**, **II**, **III**). Kogutud materjali hoiustati kuivmaterjalina temperatuuril –20 °C (**I**, **II**, **III**) või 75% etanoolis temperatuuril +4 °C või toatemperatuuril (**II**, **III**). Pistesääsklased määrati morfoloogiliste tunnuste põhjal liigi või liikide grupini, kasutades selleks üldtuntud juhendeid (**I**, **II**, **III**) (Becker jt 2010, Cranston jt 1987, Snow 1990). Hästi säilinud ja vähekulunud isendeid kasutati illustreerivate piltide tegemiseks (**III**).

Morfoloogiliste määrangute kinnitamiseks (**III**) ja pistesääsklaste geneetilise mitmekesisuse (**I**) uurimiseks tehti DNA analüüs. DNA eraldamiseks kasutati tootja juhendi järgi (**I**, **III**) komplekti DNeasy Blood & Tissue Kit (Qiagen, Hilden, Saksamaa) või vahendit PrepMan Ultra Sample Preparation Reagent (Thermo Fisher Scientific Inc., Waltham, USA) (vt Lilja jt 2018). Pistesääsklaste puhul võeti vaatluse alla COI, ND5 ja ITS2 nukleotiidijärjestused ning bakteriaalse sümbiondi *Wolbachia* tuvastamiseks kasutati sihtmärgina geeni wsp (**I**, **III**) (tabel 1).

PCRi amplifikatsioonisegu sisaldas 1 µl putuka DNAd, 12,5 µl segu DreamTaq DNA Polymerase Master Mixi (2X) (Thermo Fisher Scientific Inc. Waltham, USA), 0,5 µl kumbagi praimerit (TAG Kopenhaagen, Frederiksberg, Taani) ja ülejäänud osa nukleaasivaba vett, et tulemuseks oleks 25 µl lahust (**I**, **III**). Vajaduse järgi lisati reaktsioonisegule vee arvelt magneesiumkloriidi ($MgCl_2$) või DMSOd (**III**). Wsp geeni sekventsi paljundamisel lähtuti varem avaldatud PCRi protokollist (Shaikevich, Bogacheva ja Ganushkina 2019), kuid COI, ND5 ja ITS2 nukleotiidijärjestuste amplifitseerimiseks kasutati kohandatud programme (**I**, **III**). Geenijärjestuste paljundamise edukust kontrolliti geelelektroforeesi abil (**I**, **III**). PCRi produktide värvimiseks kasutati DNA Gel Loading Dye segu (6X) (Thermo Fisher Scientific Inc., Waltham, USA) ja amplifitseeritud DNA lõikude pikkuse hindamisel kasutati DNA markerit GeneRuler 100 bp Plus (Thermo Fisher Scientific Inc., Waltham, USA). Positiivse signaaliga PCRi proovid saadeti sekveneerimiseks Tartu Ülikooli tuumiklaborisse (Tartu Ülikool, Tartu, Eesti).

Geenijärjestuste edasi-tagasi sekventsid puhastati ja liideti BioEditi programmi versiooniga 7.2.6.1 (**I**, **III**) (Hall, 1999). Liigilise kuuluvuse tuvastamiseks võrreldi saadud järjestusi avaliku sekventsipanga teabega (**I**, **III**), kasutades selleks mõeldud otsinguprogramme BLAST (NIH, Bethesda, USA) ja BOLD Systems (Ratnasingham ja Hebert 2007). Fülogeneetiline analüüs viidi läbi MEGAXi programmi versiooniga 10.0.5 (**I**) (Kumar jt 2018). **I** artiklis võrreldi 54 Eesti pistesääsklase sekventse ning kahte Rootsist saadetud ja avalikust GenBanki infopangast (NIH, Bethesda, USA) alla laaditud 22 nukleotiidijärjestust. Sekventsid joondati funktsiooniga MUSCEL. Fülogeneetilised puud konstrueeriti suurima tõepära meetodiga, kasutades programmi soovitatud mudelit. Bootstrapi meetodi replikatsioonide arvuks määrati 1000.

II artiklis lisati igale linnakeskkonnas sooritatud putukapüügile Riigi Ilmateenistuse poolt samal ajal mõõdetud temperatuuri (°C), suhtelise niiskuse (%), tuulekiiruse (m/s) ja standardõhurõhu (hPa) tulemused. Liigilise mitmekesisuse iseloomustamiseks kasutati efektiivset liikide arvu (ENS), mis näitab, mitme võrdselt esindatud liigiga kooslusega sarnaneb päris kooslus. Välitööde loendustulemused olid Poissoni jaotusega, mistõttu dispersiooni iseloomustamiseks kasutati usaldusvahemikku nivooga 95%. Statistiliseks analüüsiks rakendati programmi R versiooni 3.6.1 (R Core Team, 2019). Kogutud andmed korrastati ja kontrolliti sõltumatute muutujate omavahelist korrelatsiooni. Lõpptulemusena kaasati statistilisse analüüsi sõltumatute muutujatena püügipunkt, -kuu ja -aasta, temperatuur, tuulekiirus ning püütud putukate sugu. Sõltumatute muutujate mõju pistesääsklaste arvukusele ja liigirikkusele hinnati üldistatud lineaarsete mudelite abil. Tähtsusetuks osutunud muutujad eemaldati mudelitest käsitsi. Kõige viimasena veenduti, et regressioonimudelites ei oleks probleeme üle- või aladispersiooni, üleliigsete nullide ega multikollineaarsusega. Programmi R kasutati ka illustreerivate jooniste loomisel.

COI, ND5 ja ITS2 markeritel põhinev analüüs näitas, et harilikul metsasääsel (*Ae. communis)* on Põhja- ja Baltimaades kaks erinevat mitokondriaalse DNA liini, kusjuures vähemlevinud liini COI markeri järjestused on palju sarnasemad Põhja-Ameerika lähiliikide omadega (**I**). Kahe geneetilise grupi eristumist toetasid mõlemad mitokondriaalsed markerid (joonis 3, joonis 4), kuid ribosomaalse DNA markeri ITS2 nukleotiidijärjestus oli kõigil hariliku metsasääse isenditel peaaegu identne (joonis 5). Seejuures erinesid COI sekventsid kahe klastri vahel keskmiselt

6,3% (±1,8%), samas kui gruppidesisene keskmine varieeruvus oli märgatavalt väiksem. Markeri ND5 puhul oli gruppidevaheline keskmine nukleotiidijärjestuste erinevus isegi 8,3% (±3,1%).

Tartu linnast koguti kolme aastaga kokku 1 890 pistesääsklast (**II**). Ühe püügikorra ajal kogutud isendite arv näitas seejuures aasta-aastalt vähenemistrendi: 2013. aastal püüti korraga keskmiselt 6,41, 95% CI [6,22–6,61] isendit, samas kui 2017. aastal koguti keskmiselt vaid 2,53, 95% CI [2,41–2,65] pistesääsklast. Kusjuures ka viimasel püügiaastal oli liikide keskmine mitmekesisus esimese aasta omast statistiliselt oluliselt kesisem. Pistesääsklaste arvukus olenes püügipunktist, aastast, kuust, temperatuurist, tuulekiirusest ning aasta ja kuu vahelisest vastastikmõjust (tabel 2). Samuti sõltus see pistesääsklaste soost ning soo ja kalendrikuu vahelisest koosmõjust. Liikide mitmekesisust mõjutasid statistiliselt oluliselt vaid püügikoht ja aasta (tabel 3). Liikide suktsessioon järgis üldiselt tüüpilist rütmi (Becker jt 2010): metsasääsed (*Aedes* spp.) võidutsesid soojaperioodi esimeses pooles ja laulusääski (*Culex* spp.) oli kõige arvukamalt suve lõpus. Siiski oli aastate lõikes näha ka mõningaid erinevusi (joonis 7).

Aastatel 2008–2020 püüti ja määrati Eestis kokku 24 344 pistesääsklast, kellest 94,24% moodustasid emased isendid (**I**, **II**, **III**). Neile andmetele, ajaloolistele andmetele (Remm 1957) ja naaberriigi muuseuminäidisele toetudes koostati ajakohastatud Eesti pistesääsklaste nimekiri (**III**) (tabel 4). Uuendatud nimekiri sisaldab 34 liiki, kellest 27 taksoni esinemine on tõestatud töö käigus püütud eksemplaridega, üks liik on lisatud naaberriigi teadlaste avaldatud teabe põhjal (Khalin ja Aibulatov 2020) ja kuus liiki põhinevad prof H. Remmi andmetel (Remm 1957). Käesoleva uurimuse tulemusena sedastati neli liiki Eesti pistesääsklaste nimekirjas esimest korda. Seejuures jäi veel mõne liigi kohalik esinemine lahtiseks, vajades edasisi uuringuid. Arvukaimaks pistesääsklaseks osutus harilik metsasääsk.

Antud uurimustöö tegemist ajendas kasvav vajadus õppida paremini tundma kohalikku pistesääsklaste faunat. Kuigi verd imevate putukate vastu on maailmas läbi aegade suurt huvi tuntud, on veel palju detaile ja seoseid, mis vajavad endiselt uurijate tähelepanu. Pistesääsklastel on märkimisväärne mõju inimeste ja loomade heaolule, kuid Eestis oli selle sugukonna uurimine kauaks soiku jäänud.

Käesolevas uurimuses näidati esimest korda, et Eesti arvukaim sääseliik, harilik metsasääsk, peidab kahte küllaltki erinevat mitokondriaalse DNA liini. Kuigi niisuguse lahknemise tekkepõhjus on veel teadmata, näitab see ilmekalt, et liikide tuvastamisel ja uute taksonite kirjeldamisel ei saa lähtuda vaid mtDNA markeritest. Küsitavuste tekkimisel peab analüüsi kaasama ka tuuma DNA lõike. Seejuures tuleb sekveneeritud järjestusi hoolikalt hinnata, sest PCRis võivad amplifitseeruda hoopis tuuma DNAsse kopeerunud mtDNA lõigud (Beebe 2018, Song jt 2008). Samuti võib tulemusi mõjutada heteroplasmia (Frey ja Frey 2004) ehk olukord, kui ühes isendis esineb mitu mtDNA versiooni (Song jt 2008).

Linna pistesääsklaste aegruumilist arvukuse ja mitmekesisuse dünaamikat ilmestas märkimisväärne varieeruvus, näidates, kui tähtis on tõepärase pildi saamiseks uurida putukapopulatsioone mitme aasta jooksul. On tähelepanuväärne, et 2013. aasta pistesääsklaste rohkele suvele eelnes lume sulamisest tingitud üleujutus ning ühtaegu soe ja niiske maikuu. Ülejäänud kahel vaatlusaastal sulas lumi juba varem ja maikuu oli kuivem. Statistiline analüüs näitas, et temperatuuri ja suhtelise niiskuse vahel valitses üldiselt negatiivne korrelatsioon. Tõenäoliselt just sellest suhtest tulenes fenomen, et temperatuuri tõustes vähenes linnast püütud pistesääsklaste hulk. See tähendab, et linnaoludes piirab vee vähesus pistesääsklaste arvukust rohkem kui temperatuur. Paljud varasemad teadustööd on näidanud, et linnakeskkond ongi mitte ainult soojem, vaid ka kuivem kui ümbritsevad alad (Araujo jt 2015, Fukui 1957). Teisalt oli tuule tugevuse negatiivne mõju pistesääsklaste arvukusele etteaimatav.

Kuigi laulusääsed (*Culex* spp.) olid igal uurimisaastal linnaoludes stabiilselt arvukad, siis kõiki Eesti püügikohti arvesse võttes moodustasid nad kogutud isenditest vaid 5,29%. See on seletatav asjaoluga, et harilik laulusääsk (*Culex pipiens* Linnaeus) ongi teadaolevalt linnakeskkonnas äärmiselt edukas (Becker jt 2010). Samas oli levinumate liikide suhteline arvukus Eesti esimeses pistesääsklaste nimekirjas ja käesolevas uurimuses äärmiselt sarnane. Pistesääsklaste kogumist ja analüüsimist tuleks jätkata, et saada parem ülevaade ka vähearvukatest liikidest ning riimveega seotud kooslustest. Samuti tuleb Eestis pöörata rohkem tähelepanu pistesääsklastega levivatele haigustekitajatele. Seejuures võib eriti tähtsaks liigiks olla just harilik metsasääsk, arvestades selle liigi suurt arvukust ja suutlikkust kanda edasi mitmeid Põhja- ja Baltimaades levivaid patogeene.

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I

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Novel Mitochondrial DNA Lineage Found among *Ochlerotatus communis* (De Geer, 1776) of the Nordic-Baltic Region

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Abstract: The *Ochlerotatus (Oc.) communis* complex consist of three Northern American species as well as a common Holarctic mosquito (Diptera: Culicidae) *Oc. communis* (De Geer, 1776). These sister species exhibit important ecological differences and are capable of transmitting various pathogens, but cannot always be differentiated by morphological traits. To investigate the *Oc. communis* complex in Europe, we compared three molecular markers (COI, ND5 and ITS2) from 54 Estonian mosquitoes as well as two COI marker sequences from Sweden. These sequences were subjected to phylogenetic analysis and screened for *Wolbachia* Hertig and Wolbach symbionts. Within and between groups, distances were calculated for each marker to better understand the relationships among individuals. Results demonstrate that a group of samples, extracted from adult female mosquitoes matching the morphology of *Oc. communis*, show a marked difference from the main species when comparing the mitochondrial markers COI and ND5. However, there is no variance between the same specimens when considering the nuclear ITS2. We conclude that *Oc. communis* encompasses two distinct mitochondrial DNA lineages in the Nordic-Baltic region. Further research is needed to investigate the origin and extent of these genetic differences.

Keywords: *Ochlerotatus churchillensis*; *Ochlerotatus nevadensis*; *Ochlerotatus tahoensis*; barcoding; phylogenetics; speciation; vectors

1. Introduction

Ochlerotatus (*Oc.*) *communis* complex includes four closely related mosquito species [1]: *Oc. communis* (De Geer, 1776), *Oc. churchillensis* (Ellis and Burst, 1973), *Oc. nevadensis* (Chapman and Barr, 1964) and *Oc. tahoensis* (Dyar, 1916). Morphology-based delimitation of these species is highly problematic due to a lack of reliable distinguishing traits, especially in adults [2]. Thus, researchers have employed both DNA sequencing, using mainly the mitochondrial cytochrome c oxidase subunit I (COI or COX1), and restriction fragment length polymorphism (RFLP) patterns to help with differentiation [3]. The namesake of the group, *Oc. communis*, is a common and often numerous Holarctic pest, whereas the other three species appear to be native to Northern America [1]. Due to the ubiquity of *Oc. communis* and because of its observed morphological variability, it is highly likely that this complex could have additional sister species in other parts of the world besides Northern America [2].

The phylogeography of the *Oc. communis* complex has received relatively little attention. At first these species were distinguished by morphologic as well as morphometric traits and the length differences of select loci, apparent in electrophoresis [1,4]. In 2014, the journal *Canadian Entomologist* published an article from H. H. Namin et al. describing barcoding (COI) results and designing a new diagnostic RFLP pattern for use with *Oc. communis*, *Oc. churchillensis* and *Oc. tahoensis* [3]. Since that, *Oc. churchillensis*, *Oc. nevadensis* and *Oc. tahoensis* have only been rarely sequenced, for example as part of vector disease investigations [5]. There are more studies on *Oc. communis*, but this species is still often diagnosed based on morphology alone, although genetic identification is also used [6–10]. Generally speaking, the *Oc. communis* complex does not currently appear to be under close study.

Mosquitoes from the *Oc. communis* complex have been associated with many pathogens. The Jamestown Canyon virus has been isolated from North American *Oc. communis* mosquitoes, which may be one of the species acting as an overwintering reservoir for the pathogen [5]. *Oc. communis* individuals, in some cases both adults and larva, have been found to carry Sindbis virus (known in Sweden as Ockelbo, in Finland as Pogosta and in Russia as Karelian virus) Batai virus, *Francisella (F.) tularensis* bacteria as well as different strains of the Inkoo virus in Scandinavian field studies [7,11,12]. *Oc. communis* could also be one of the main vectors of *Dirofilaria repens*, a filarial nematode which is currently expanding its area northward [8,13]. According to older studies, *Oc. communis* mosquitoes have also tested positive for Tahyna virus in Russia and six strains of California encephalitis virus in Canada [14,15].

Individuals within a phylogenetic group, even within isomorphic species, can often differ in their medical importance [16,17] and there is a noticeable lack of information regarding how the sister species within *Oc. communis* complex vary in their vector capacity and competence. Especially as some biological and ecological differences have been observed within the group. Firstly, *Oc. churchillensis* is the only autogenous species in the group and thought to be non-biting [1]. Both *Oc. nevadensis* and *Oc. tahoensis* seem to only be found in mountainous regions, the latter preferring higher elevations, while *Oc. churchillensis* inhabits forests near the North American tundra [4]. Because of these factors, it has been theorized that the sister species comprising *Oc. communis* complex may have derived from allopatric as well as sympatric speciation [3].

Maternally inherited *Wolbachia* Hertig and Wolbach, 1924 symbionts can also contribute to speciation within arthropods. *Wolbachia* is a genus of cytoplasmically transmitted bacteria that infect the tissues of many arthropods and some nematodes [18,19]. These endosymbionts have shown to cause cytoplasmic incompatibility, parthenogenesis and the death or feminization of biological males (reviewed by Correa and Ballard [20]). Because of this, *Wolbachia* infections have been seen as possible drivers of microevolution and even speciation [21–23]. *Wolbachia* strains have been detected in several different mosquito species, but infection rates vary [24,25]. At this time, no strains have been found in *Oc. communis* [8]. However, if detected, it could help explain some genetic results.

A larva with a COI sequence similar but not identical to *Oc. tahoensis* was recently found in Sweden [7], but the discovery was not further investigated. At the same time, similar cases were found with adult female mosquitoes in Estonia. Taking into account the possibility of additional *Oc. communis* complex species in Europe [2], a special attention was paid to the recently collected Estonian mosquitoes. The primary aim of this study was to search for a possible novel species within the *Oc. communis* complex. For this reason, both mitochondrial (mtDNA) and nuclear DNA (nDNA) markers from Nordic-Baltic mosquitoes were analyzed. These samples were also screened for infection with *Wolbachia* symbionts. Here we present the genetic information of 54 Estonian mosquitoes and compare those to two Swedish samples as well as to reference material from public nucleotide databases.

2. Materials and Methods

2.1. Sampling and Morphological Identification

This study is based on 54 Estonian mosquitoes and two Swedish COI sequences (Table A1). Of the Estonian individuals, 26 were morphologically identified as *Oc. communis*, six as *Oc. punctor* (Kirby,

1837), eight as *Oc. hexodontus* (Dyar, 1916), 13 as *Oc. cataphylla* (Dyar, 1916) and one as *Anopheles (An.) messeae* (Falleroni, 1926). While *Oc. communis* is the main focus, *Oc. punctor*, *Oc. hexodontus* and *Oc. cataphylla* samples were included in the analysis to compare how intra- as well as interspecific genetic variation of the *Oc. communis* complex relates to other common species of the genus *Ochlerotatus*. *An. messeae* was used as an outgroup. All Estonian mosquitoes were collected from six different sites during 2015–2016, using automated Mosquito Magnet® Independence (Woodstream Corp., Lititz, PA, USA) machines. Four of these sampling sites were located on the Estonian mainland and two on the largest islands of the country—Saaremaa and Hiiumaa (Figure 1). Insects were stored at −20 °C until temporarily thawed and identified under a stereomicroscope Olympus SZ61 (Olympus Corporation, Shinjuku, Tokyo, Japan) to species level, using a standard taxonomic key [2]. Swedish COI sequences originate from Västerbotten County, first from a larva caught in 2014 [7] and the second from an adult female mosquito sent to the Swedish National Veterinary Institute (SVA) in 2017.

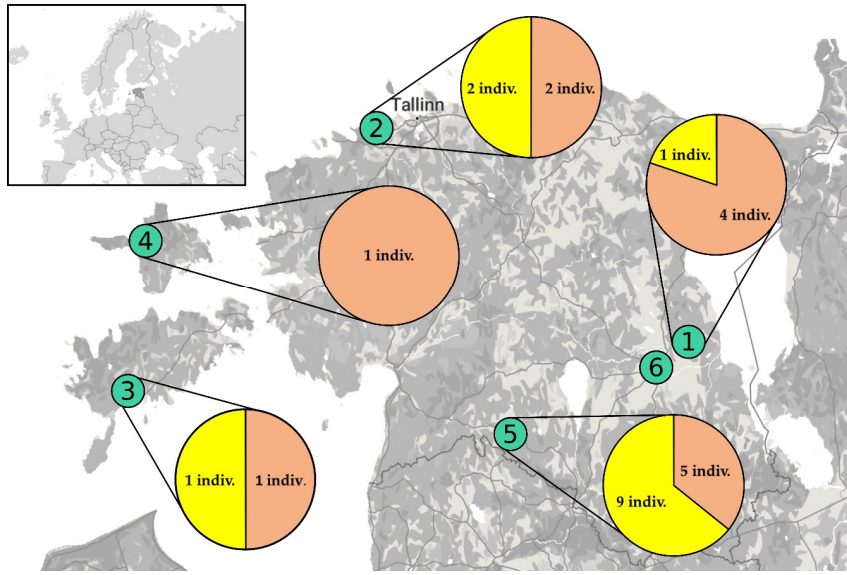

Figure 1. Map showing the six Estonian collection sites, indicated with green circles. Pie charts demonstrate the amount of *Oc.* sp. (yellow background) and *Oc. communis* (orange background) specimen caught from each site. In total this paper includes 20 mosquitoes from site 1 "Undi" (58°29′ N, 26°54′ E), eight from site 2 "Muraste" (59°28′ N, 24°27′ E), four from site 3 "Mändjala" (58°13′ N, 22°20′ E), five from site 4 "Vanajõe" (58°53′ N, 22°26′ E), 16 specimens from site 5 "Metsaküla" and one mosquito from site 6 "Tartu" (58°04′ N, 25°31′ E). 31 of these mosquitoes were caught in 2015 and 23 during 2016. Base map curtesy of ©OpenStreetMap contributors (https://www.openstreetmap.org/copyright) and ©MapTiler (https://www.maptiler.com/copyright/).

2.2. DNA Extraction

DNA was extracted from the Estonian mosquitoes using either DNeasy Blood and Tissue Kit (Qiagen, Hilden, North Rhine-Westphalia, Germany) or PrepMan® Ultra Sample Preparation Reagent (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). There were no qualitative differences between the used DNA extraction methods. Whole mosquitoes were used for the DNeasy Blood and Tissue Kit, while three legs from each specimen were taken for extracting DNA with the PrepMan® Ultra Sample Preparation Reagent. DNeasy Blood and Tissue Kit was used in accordance with the

manufacturers manual and the DNA extractions made with PrepMan® Ultra Sample Preparation Reagent were conducted as specified in previous work [26].

2.3. DNA Markers

Two protein coding mitochondrial and two nuclear markers were amplified and sequenced from all Estonian mosquitoes used in this study. However, only three markers were used for further analysis as the D2 region of the large subunit 28S rDNA gene was too conserved between *Ochlerotatus* species to be of use, although it has been successfully utilized for species identification in other mosquito genera [27,28]. The 5′ region of the cytochrome c oxidase (COI) subunit I was chosen as one of the markers for its widespread use in mosquito identification and its generally good ability to differentiate between species, although it can at times either over- or underestimate the true number of well distinguished monophyletic groups [16,29,30]. The nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 5 (ND5) gene was used as an additional mitochondrial marker. ND5 is generally thought to have faster evolution rates compared to COI and thus has been used for inter- as well as intraspecies studies in mosquitoes [31–33]. Finally, the nuclear internal transcribed spacer 2 (ITS2), a region of the ribosomal RNA gene, was used as the most conserved marker. ITS2 sequences have been used for species identification in many animal groups and may be the most used nDNA marker for mosquitoes [16,34,35]. It has generally been proposed as a good marker to analyze alongside COI [16].

Each mosquito sample was also screened for *Wolbachia* symbionts by amplifying part of the *Wolbachia* surface protein (WSP) gene.

2.4. Primers

For COI, which is by far the most commonly sequenced marker for mosquitoes, we used the universal primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTG G-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′), that consistently resulted in 710 bp long sequences [36]. A 450 bp segment of the ND5 region was amplified and sequenced using the primers 6500 (5′-TCCTTAGAATAAAATCCCGC-3′) and 7398 (5′-GTTTCTGCTTTAGTTCATTCTTC-3′) which were originally designed for *Aedes (Ae.) albopictus* [37]. Primer pair 5.8S (5′-TGTGAACTG CAGGACACATG-3′) and 28S (5′-ATGCTTAAATTTAGGGGGTA-3′) was used for ITS2, producing approximately 368 bp to 387 bp long sequences [38]. The ITS2 primers were initially developed to differentiate between cryptic Anopheline mosquitoes, but can be used for other mosquito genera as well. DNA from the *Wolbachia* symbionts WSP gene was amplified with the primers wsp 81F (5′-TGGTCCAATAAGTGATGAAGAAAC-3′) and wsp 691R (5′-AAAAATTAAACGCTACTCCA-3′) that would have resulted in 600 bp sequences [39].

2.5. Polymerase Chain Reaction (PCR) and Sequencing

While the composition of the polymerase chain reaction (PCR) mixes remained the same throughout, thermal cycler programs were adjusted for each primer pair to maximize the amplification yield of the respective marker regions. All PCR reactions contained 12.5 µL DreamTaq DNA Polymerase Master Mix (2X) (Thermo Fisher Scientific Inc., Waltham, MA, USA), 0.4 µM of each primer (0.04 µmol, TAG Copenhagen, Frederiksberg, Denmark), 10.5 µL nuclease-free water and 1 µL DNA template. The PCR program for amplifying the COI marker region was as follows: 95 °C for 2 min 15 s, followed by 35 cycles of 95 °C for 30 s, 57 °C for 45 s, 72 °C for 45 s and a final elongation step at 72 °C for 5 min. Although mostly identical, the PCR program for the ITS2 sequence introduced a much lower annealing temperature: 95 °C for 2 min 15 s, 35 cycles of 95 °C for 30 s, 45 °C for 45 s, 72 °C for 45 s and an elongation at 72 °C for 5 min. On the other hand, amplifying the mitochondrial ND5 region required a much longer PCR program, which contained 2 different sets of cycles on low annealing temperatures. The final program was: 94 °C for 3 min denaturation followed by 10 cycles of 94 °C for 30 s, 38 °C for 30 s, 65 °C for 45 s, then 50 cycles of 94 °C for 30 s, 38 °C for 30 s, 65 °C for 45 s and a last elongation of 65 °C for 3 min. The WSP region of the symbiont was amplified using a previously published PCR

program [24] and a positive control sample from *Culex pipiens* was also added to the PCR. All samples were amplified with ESCO Swift Maxi Thermal Cycler (ESCO Micro Pte. Ltd., Changi South Street, Singapore, Singapore).

PCR products were checked for signals by electrophoresis using 1.6% agarose gel infused with ethidium bromide. Amplified samples were tinted with DNA Gel Loading Dye (6X) (Thermo Fisher Scientific Inc., Waltham, MA, USA) prior to electrophoresis. Positive signals were compared to GeneRuler 100 bp DNA Ladder, ready-to-use (Thermo Fisher Scientific Inc., Waltham, MA, USA) or, in the case of the WSP samples, to the GeneRuler 100 bp Plus DNA Ladder, ready-to-use (same company) to visually determine the approximate lengths of the replicated DNA strands. PCR products were cleaned and sequenced with Applied Biosystems 3130xl Genetic Analyzer by a two-directional procedure (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.6. Sequence Analysis

Forward and reverse DNA strands were aligned and assembled into consensus sequences in BioEdit Sequence Alignment Editor version 7.2.5 [40]. Primers and low-quality areas were trimmed to produce the final sequences. MEGA X version 10.0.5 [41] was used for sequence alignment and data analysis. All original DNA sequences were uploaded to the online database GenBank. Reference sequences were added to the analysis via the Blast Search tool in MEGA X (Table A2). This was effective for COI and ITS2 markers. However, the mtDNA marker ND5 has so far received little attention in mosquitoes from the *Ochlerotatus* genus and, therefore, no previous sequences could be found from the database.

Protein-coding gene sequences were aligned based on codons, whereas the DNA strands for ITS2 were aligned by nucleotides alone and allowed gaps for indels. In all cases, the Multiple Sequence Comparison by Log-Expectation (MUSCEL) tool with default options was used for aligning sequences. The Find Best-Fit Substitution Model feature was used for all of the analyzed markers to determine the most appropriate model and Rates among Sites variable. These results were then used to calculate mean within and between group genetic distances measuring the proportion of nucleotide sites with differences between each sequence pair (p-distances). This was done using the Compute within Group Mean Distance and Compute between Group Mean Distance functions in MEGAX. Gaps/Missing Data Treatment was set to complete deletion, which ensured that all sequences of the same marker were trimmed to identical lengths: 441 bp for COI, 321 bp for ND5 and 251 bp for the ITS2 marker region.

Phylogenetic trees were constructed with the maximum likelihood method, while also using the analysis model and Rates among Sites recommended by the Find Best-Fit Substitution Model feature. The Gamma Parameter (+G) was set to 6, Gaps/Missing Data Treatment was set to complete deletion and Bootstrap with 1000 replications was employed each time. All trees were annotated and rooted using *An. messeae* as an outgroup. Trees were then modified to only display bootstrap values > 75% and distance values ≥ 0.01. Differences between population sizes were not accounted for. While *Oc. communis*, *Oc. punctor* and *Oc. cataphylla* are all common in Estonia, there is very little information about their exact effective population sizes. However, mosquitoes of the *Oc.* sp. group likely have a much smaller effective population size than the normal type *Oc. communis*.

3. Results

Of the 54 Estonian mosquitoes used in this study, 26 were identified as *Oc. communis* by morphological evaluation, but only 14 of these were grouped together by all three DNA markers. The remaining 12 individuals formed a separate monophyletic group within the *Oc. communis* complex based to their mitochondrial markers, similarly to two COI sequences received from Sweden. However, this pattern was not apparent when examining the nDNA results.

According to the phylogenetic tree based on the COI marker (Figure 2), 12 sequences from mosquitoes caught at three different sites (sites 1, 2 and 5) in Estonia (Figure 1) and 2 sequences from Sweden cluster together (hereafter referred to as *Oc.* sp.), distinct from *Oc. communis* and closer to the

North American species *Oc. tahoensis* and *Oc. churchillensis*. In fact, there is on average about 0.063 (standard error (S.E) 0.018) substitutions per base difference between *Oc. communis* specimens and the *Oc.* sp. group. Meanwhile, the p-distances between *Oc.* sp. individuals and *Oc. tahoensis* as well as *Oc. churchillensis* are smaller, 0.046 (S.E. 0.013) and 0.054 (S.E. 0.015) respectively. It should also be noted, that the *Oc.* sp. COI sequences show a high similarity to each other, being almost genetically identical. This is in contrast to the larger genetic diversity within other groups. These results show that there is a previously unknown group of genetically distinct individuals belonging to the *Oc. communis* complex found in Europe.

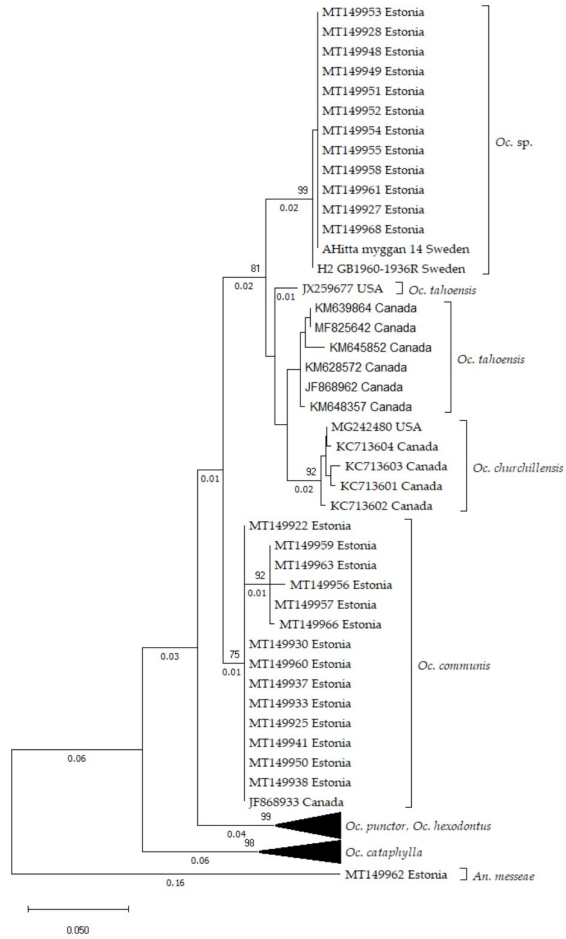

Figure 2. Phylogenetic tree based on 72 partial cytochrome c oxidase (COI) sequences (441). Calculated with the Maximum Likelihood method, using Tamura 3-parameter [42] model, with discrete gamma distribution (6 categories (+G, parameter = 0.1790)) Branch lengths are shown to scale and measured as the number of substitutions per site. *An. messeae* was used as an outgroup for rooting the tree. *Oc.* sp. sequences are genetically closer to North American species *Oc. tahoensis* and *Oc. churchillensis* than the widespread *Oc. communis*.

The ND5 marker region also suggests that mosquitoes forming the *Oc.* sp. group are a distinct genetic unit, separate from traditional *Oc. communis* mtDNA sequences (Figure 3). Compared to the COI region, the ND5 marker sequences are even more variable between groups. Between *Oc. communis* and the *Oc.* sp. cluster, there is a difference of on average 0.083 (S.E 0.031) base substitutions per site. Yet, within group average evolutionary distances remain small. In the *Oc.* sp. group, there are only on average 0.001 (S.E 0.001) base substitutions per site over all of the sequence. The p-distance is once again larger among the traditional *Oc. communis* samples, averaging 0.008 (S.E 0.009) base substitutions. ND5 marker sequences associated with *Oc. punctor*, *Oc. hexodontus* and *Oc. cataphylla* clusters are even more variable. Unfortunately, there are no ND5 marker sequences from *Oc. tahoensis*, *Oc. churchillensis* or *Oc. nevadensis* currently available in GenBank. Likewise, because the ND5 region is a less popular marker than COI and ITS2, there were also no reference sequences for *Oc. communis*, *Oc. punctor*, *Oc. hexodontus* or *Oc. cataphylla*. However, the ND5 sequences included in this study support the conclusions drawn from the COI marker.

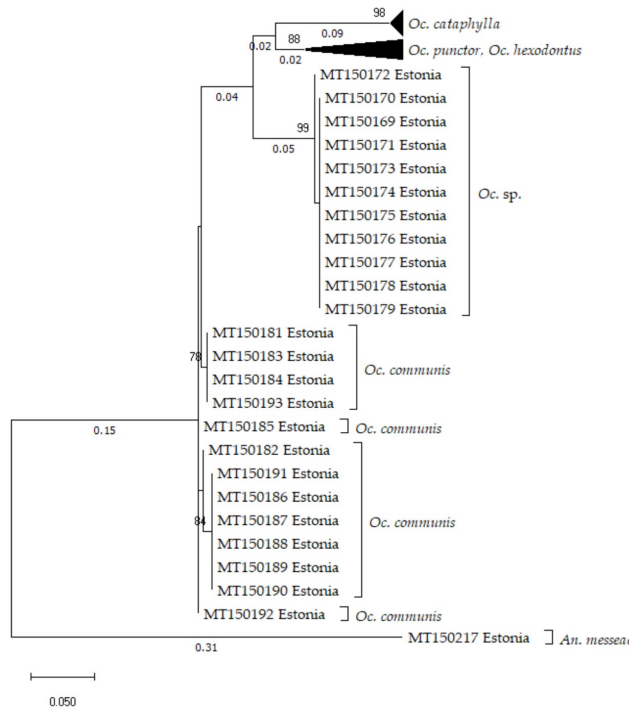

Figure 3. Phylogenetic tree representing the information of 48 dehydrogenase subunit 5 (ND5) marker region sequences (321 bp). The tree is calculated with the maximum likelihood method, using the Tamura 3-parameter [42] model and a discrete gamma distribution (6 categories (+G, parameter = 0.1516)). Branch lengths are shown to scale and measured based on the average number of substitutions per site between sequence pairs. *An. messeae* was used the outgroup in order to root the tree. *Oc.* sp. sequences cluster together, away from the *Oc. communis* group.

While mitochondrial markers outlined *Oc.* sp. group as a separate entity, this is not the case for the nuclear marker ITS2. In fact, there are no differences between the ITS2 sequences from *Oc. communis* and the *Oc.* sp. samples (Figure 4). On the other hand, *Oc. churchillensis* reference

sequences downloaded from GenBank maintain their genetic distance from *Oc. communis*, being on average 0.025 (S.E. 0.009) substitutions per base apart. The same is true for *Oc. punctor*, *Oc. hexodontus* and *Oc. cataphylla* clusters, which differ on average from the *Oc. communis* group by 0.035 (S.E 0.011) and 0.079 (S.E 0.017) base substitutions per site, respectively. It should also be noted that the ITS2 sequences of the *Oc. communis* cluster have no notable within-group genetic variance. Within group average genetic variance is also relatively low in other groups. From these results we can see that the ITS2 region of Estonian *Oc. communis* individuals is quite conserved and does not echo the variance shown by the mtDNA markers.

Figure 4. Phylogenetic tree based on the 53 internal transcribed spacer 2 (ITS2) marker sequences (251 bp). Calculated with the maximum likelihood method, using the Kimura 2-parameter model [43] and discrete Gamma distribution (6 categories (+G, parameter = 0.8737)). Branch lengths are shown to scale and measured based on the average number of substitutions per site between sequence pairs. *An. messeae* was used as the outgroup for rooting the tree. There appears to be no variation amid the ITS2 sequences *Oc.* sp. sequences and *Oc. communis*. However, *Oc. churchillensis* sequences obtained from GenBank remain as a separate group.

Estonian mosquito samples were screened for *Wolbachia* surface protein as it could offer an explanation for the divergent mtDNA lineage within the analyzed *Oc. communis* individuals. However, there were no positive electrophoresis signals for any of the samples except for the positive control. Therefore, there is no evidence of *Wolbachia* symbionts in the *Oc. communis* individuals used in this study.

4. Discussion

There is a discrepancy between the mtDNA and nDNA markers among mosquitoes morphologically identified as *Oc. communis* in the Nordic-Baltic region. The mitochondrial markers COI and ND5 clearly distinguish two clusters of individuals within the *Oc. communis* complex: One identical to *Oc. communis* reference sequences and the other representing a new group. However, this difference is not reflected in the ITS2 sequences of the same mosquitoes. Based on the nuclear marker, all examined *Oc. communis*-like individuals have identical ITS2 marker sequences, whereas reference sequences for *Oc. churchillensis*, which also belongs to the *Oc. communis* complex, remain distinct from *Oc. communis*. There appears to be a previously unreported mtDNA lineage within the Nordic-Baltic *Oc. communis* populations, but no variance within the nDNA to point to a distinct species.

Both COI and ND5 marker regions have high support for similar monophyletic clades and both trees show some amount of nucleotide changes between traditional *Oc. communis* individuals, while *Oc.* sp. sequences are largely identical. This shows, that the *Oc.* sp. clade is evolutionarily younger than the traditional *Oc. communis* and its members probably less numerous, as the mtDNA of this group has yet to accrue many mutations [44]. However, the nuclear marker ITS2 shows noticeably less divergence between all of the analyzed *Ochlerotatus* species. Moreover, the D2 region of the large subunit 28S, also originally sequenced for this study, was unable to provide any notable differences within *Ochlerotatus*, although it has been shown to work for anopheline mosquitoes [28]. It seems that the species analyzed for this study are in general more closely related to each other than those most often sequenced in other mosquito genera. Therefore, it is currently difficult to say if any of the markers used in this work might underestimate the true diversity of the *Ochlerotatus* species. This matter would be greatly improved by finding and analyzing nDNA markers with better resolution for the *Ochlerotatus* genus. It would also be useful to sequence a larger number of *Oc. communis*, in order to obtain a better overview of its natural intraspecific variance.

The reason for mitochondrial differences between coexisting groups can be hard to pinpoint. As the common type *Oc. communis* and the individuals with differing mtDNA coexist in the same communities and have identical ITS2 sequences, it is probable that they are intermixing. MtDNA variation in arthropod populations can be influenced by symbiotic bacteria like the maternally inherited *Wolbachia* [45]. However, none of the samples used in this study were positive for *Wolbachia* DNA and this coincides with previous observations [8]. There could be other speciation-driving factors in play, but that is impossible to determine at this juncture. Importantly, the fauna of the Nordic-Baltic region is relatively young, only emerging after the Last Glacial Period more than 9000 years ago when it was recolonized by organisms from different glacial refugiums [46–48]. It is possible that Eurasian *Oc. communis* complex individuals with the differing mtDNA used to be geographically separated, but not genetically different enough as to have evolved reproductive barriers.

Uncertainties in mtDNA sequencing results can also be caused by pseudogenes or heteroplasmy. Many organisms are known to have nuclear insertions of mitochondrial sequences (NuMts) as well as multiple types of functional mtDNA in the same individual (heteroplasmy)—both of these can interfere with the amplification and sequencing of mitochondrial markers, resulting in erroneous conclusions if not recognized [49]. Sequences from COI and ND5 genes have been known to be incorporated into nDNA as NuMts [50]. Furthermore, starting with *Aedes aegypti* (Linnaeus, 1762) and *Culex quinquefasciatus* (Say, 1823), NuMts have now been found in many mosquito species [51,52]. However, these insertions in nDNA are non-functional and, therefore, not under the same constraints or mutation rates as the real mtDNA [53,54]. These pseudogenes tend to contain inappropriate stop-codons and indels as well as more point mutation than normal [49,55]. Such problems are not

evident in the sequences analyzed in this study. Heteroplasmy can also result in ambiguous reads in Sanger sequencing, but can be harder to identify compared to NuMts [16]. It cannot be completely ruled out that the less common *Oc. communis* mtDNA lineage reported in this study is a sign of heteroplasmy.

While *Oc. communis* is not currently considered an important disease vector, it has been indicated as a possible carrier of several viral and bacterial pathogens. This species is known for its wide distribution and can be numerous at times [2]. Because of this, genetic deviations within *Oc. communis* populations may be important from future vector and pest control standpoints. Estonian and Swedish faunas are relatively young and it is reasonable to assume that *Oc. communis* individuals carrying the different mtDNA variant are not limited to the Nordic-Baltic region, but can be found within a much wider area. However, there has thus far been little indication of notable genetic discrepancies within this species in Central and Western Europe. Because of this, more sampling efforts should be directed towards Eastern Eurasia. Also, there is more work to be done in regards to sequencing additional nDNA markers from *Oc. communis* complex species and finding more informative nDNA markers to use with genus *Ochlerotatus* in general.

5. Conclusions

The current study presents evidence for an additional discrete mtDNA lineage within *Oc. communis* in Europe. This common Holarctic pest is the namesake of the *Oc. communis* complex, which it shares with three closely related Northern American species. While these sister species have sometimes been regarded as subspecies to *Oc. communis*, there are clear differences in their ecology, genetic material and in some cases morphology. It has been long theorized that there could be more species belonging to the *Oc. communis* complex in other parts of the world besides Northern America. In this study we show, based on the COI and ND5 markers, that there is a group of *Oc.* sp. individuals with a distinct mtDNA lineage within morphologically identified *Oc. communis* mosquitoes in Estonia and Sweden. We also show that these differences are not apparent in the nDNA of the same individuals. It was also determined that the analyzed mosquitoes had no detectable *Wolbachia* infection, ruling these maternally inherited symbionts out as a possible explanation for the mitochondrial differences.

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Appendix A

Table A1. Mosquitoes used in this study with their GenBank sequence numbers and upload year where possible as well as the country of origin.

ID	Site No.	Morphologically Determined Species	Country of Origin	GenBank COI Acc. Num.	GenBank ND5 Acc. Num.	GenBank ITS2 Acc. Num.	Upload Year
15004012	1	*Oc. hexodontus*	Estonia	MT149916	MT150197	MT150247	2020
15004015	1	*Oc. cataphylla*	Estonia	MT149946	MT150204	MT150252	2020
15005002	1	*Oc. punctor*	Estonia	MT149917	MT150196	-	2020
15005005	1	*Oc. punctor*	Estonia	MT149918	-	-	2020
15005007	1	*Oc. hexodontus*	Estonia	MT149919	MT150198	-	2020
15005008	1	*Oc. cataphylla*	Estonia	MT149920	MT150205	MT150253	2020
15005010	1	*Oc. cataphylla*	Estonia	MT149921	MT150206	MT150254	2020
15005012	1	*Oc. communis*	Estonia	MT149922	MT150192	MT150230	2020
15009025	2	*Oc. cataphylla*	Estonia	MT149923	MT150207	MT150255	2020
15009026	2	*Oc. cataphylla*	Estonia	MT149924	MT150208	-	2020
15009027	2	*Oc. communis*	Estonia	MT149925	MT150184	MT150221	2020
15009028	2	*Oc. punctor*	Estonia	MT149926	MT150195	MT150243	2020
15009030	2	*Oc.* sp.	Estonia	MT149927	MT150171	MT150234	2020
15009031	2	*Oc.* sp.	Estonia	MT149928	MT150172	MT150235	2020
15009036	2	*Oc. hexodontus*	Estonia	MT149929	MT150199	MT150248	2020
15010004	2	*Oc. communis*	Estonia	MT149930	MT150185	MT150222	2020
15013001	1	*Oc. cataphylla*	Estonia	MT149931	MT150209	MT150256	2020
15013003	1	*Oc. cataphylla*	Estonia	MT149932	MT150210	MT150257	2020
15013004	1	*Oc. communis*	Estonia	MT149933	MT150183	MT150220	2020
15013005	1	*Oc. cataphylla*	Estonia	MT149934	MT150211	MT150258	2020
15013006	1	*Oc. punctor*	Estonia	MT149935	MT150194	-	2020
15013015	1	*Oc. cataphylla*	Estonia	MT149936	MT150212	MT150259	2020
15016024	3	*Oc. communis*	Estonia	MT149937	MT150182	MT150219	2020
15016030	3	*Oc. communis*	Estonia	MT149938	MT150181	MT150218	2020
15016041	3	*Oc. hexodontus*	Estonia	MT149939	MT150200	MT150249	2020
15018001	4	*Oc. hexodontus*	Estonia	MT149940	-	MT150245	2020
15018007	4	*Oc. communis*	Estonia	MT149941	MT150186	MT150223	2020
15018008	4	*Oc. hexodontus*	Estonia	MT149942	-	MT150246	2020
15018011	4	*Oc. punctor*	Estonia	MT149943	-	MT150244	2020
15020002	3	*Oc. cataphylla*	Estonia	MT149944	MT150213	MT150260	2020
15032002	4	*Oc. cataphylla*	Estonia	MT149945	MT150214	MT150261	2020
16008001	6	*Oc. cataphylla*	Estonia	MT149947	MT150215	MT150262	2020
16079095	5	*Oc.* sp.	Estonia	MT149948	MT150173	MT150236	2020
16079097	5	*Oc.* sp.	Estonia	MT149949	MT150169	MT150232	2020
16079102	5	*Oc. communis*	Estonia	MT149950	-	MT150224	2020
16079110	5	*Oc.* sp.	Estonia	MT149951	MT150170	MT150233	2020
16079112	5	*Oc.* sp.	Estonia	MT149952	MT150174	MT150237	2020
16079120	5	*Oc.* sp.	Estonia	MT149953	MT150175	MT150238	2020
16142001	5	*Oc.* sp.	Estonia	MT149954	MT150176	MT150239	2020
16142002	5	*Oc.* sp.	Estonia	MT149955	MT150177	MT150240	2020
16142005	5	*Oc. cataphylla*	Estonia	MT149969	MT150216	MT150263	2020
16142006	5	*Oc. communis*	Estonia	MT149956	MT150187	MT150225	2020
16142007	5	*Oc. communis*	Estonia	MT149957	MT150188	MT150226	2020
16142010	5	*Oc.* sp.	Estonia	MT149958	MT150178	MT150241	2020
16142013	5	*Oc. communis*	Estonia	MT149959	MT150189	MT150227	2020
16142016	5	*Oc. communis*	Estonia	MT149960	MT150193	MT150231	2020
16142023	5	*Oc.* sp.	Estonia	MT149961	MT150179	MT150242	2020
16142040	5	An. messeae	Estonia	MT149962	MT150217	MT150264	2020
16149001	1	*Oc. communis*	Estonia	MT149963	MT150190	MT150228	2020
16149003	1	*Oc. hexodontus*	Estonia	MT149964	MT150202	MT150250	2020
16149004	1	*Oc. punctor*	Estonia	MT149965	MT150203	-	2020
16149006	1	*Oc. communis*	Estonia	MT149966	MT150191	MT150229	2020
16149007	1	*Oc. hexodontus*	Estonia	MT149967	MT150201	MT150251	2020
16149009	1	*Oc.* sp.	Estonia	MT149968	-	-	2020
H2_GB1960-1936R	-	N/A	Sweden	-	-	-	-
hitta_myggan_14	-	N/A	Sweden	-	-	-	-

Table A2. GenBank reference sequences used in the phylogenetic analysis, selected based on their similarity to the Swedish and Estonian mosquito sequences.

Species	Country of Origin	GenBank COI Acc. Num.	GenBank ND5 Acc. Num.	GenBank ITS2 Acc. Num.	Upload Year
Oc. tahoensis	USA	JX259677	-	-	2012
Oc. churchillensis	USA	MG242480	-	-	2018
Oc. tahoensis	Canada	JF868962	-	-	2018
Oc. communis	Canada	JF868933	-	-	2018
Oc. punctor	Belgium	KM258280	-	-	2015
Oc. hexodontus	Canada	KR697054	-	-	2018
Oc. cataphylla	Sweden	KP942759	-	-	2018
Oc. tahoensis	Canada	KM648357	-	-	2019
Oc. tahoensis	Canada	KM628572	-	-	2019
Oc. churchillensis	Canada	KC713604	-	-	2013
Oc. tahoensis	Canada	MF825642	-	-	2018
Oc. tahoensis	Canada	KM645852	-	-	2019
Oc. tahoensis	Canada	KM639864	-	-	2019
Oc. churchillensis	Canada	KC713602	-	-	2013
Oc. churchillensis	Canada	KC713603	-	-	2013
Oc. churchillensis	Canada	KC713601	-	-	2013
Oc. communis	Canada	-	-	KF535022	2013
Oc. churchillensis	Canada	-	-	KF535013	2013
Oc. churchillensis	USA	-	-	MG232613	2018
Oc. punctor	Canada	-	-	KF535072	2013
Oc. hexodontus	Canada	-	-	KF535039	2013
Oc. abserratus	Canada	-	-	KF535026	2013

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Friends in All the Green Spaces: Weather Dependent Changes in Urban Mosquito (Diptera: Culicidae) Abundance and Diversity

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Simple Summary: Many female mosquitoes require vertebrate blood for egg production. Cities are becoming increasingly important points of contact between mosquitoes and their prey, as large-scale urbanization continues. Human settlements represent unique but fragmented habitats that are permanently warmer than rural areas. Because of this, there is a growing demand to better understand urban mosquito populations and the factors affecting them in various circumstances. The aim of this study was to investigate the weather conditions influencing mosquito species and abundance in a Northern European town. Thus, a three-year-long mosquito collection effort was undertaken in Estonia. Results indicated that the number of active mosquitoes decreased with wind and higher temperatures. Interestingly, there was a significant negative correlation between temperature and humidity. Furthermore, while mosquitoes belonging to the *Culex pipiens*/*Culex torrentium* group were consistently abundant during the end of the warm season, other dominant species varied considerably between the months and the three study years. Overall, springtime hydrological conditions seemed to greatly influence the mosquito season. Urbanization could generate both higher temperatures and drier environments, resulting in fewer mosquitoes in some areas. This study also revealed the mosquito species most likely to contribute to disease transmission in Estonian towns.

Abstract: Mosquitoes (Diptera: Culicidae) are universally recognized as troublesome pests and vectors of various pathogens and parasites. Understandably, the species makeup and diversity of individual populations depends on local and broad scale environmental trends, especially on temperature and hydrological variations. Anthropogenic landscapes make for unique habitats, but their effect on insects likely varies across climatic regions. The aim of this study was to investigate the diversity and seasonal patterns of urban mosquitoes in the boreal region. Specimens were collected with an insect net from May to September during three years and determined to species or species group level. Weather information was added to each data point and results analyzed using multivariate regression models. Fieldwork yielded 1890 mosquitoes from four genera. Both abundance and the effective number of species (ENS) significantly decreased during the study period. The number of collected mosquitoes had a negative correlation with wind speed and temperature, latter of which exhibited a negative association with humidity. Species succession followed predictable patterns, but with some variation between years. Still, *Culex pipiens*/*Culex torrentium* were the most abundant throughout the study. Importantly, all dominant species were known disease vectors. Our work showed that higher temperatures could result in fewer mosquitoes in boreal towns.

Keywords: *Aedes*; *Anopheles*; *Coquillettidia*; *Culex*; *Culiseta*; entomology; Estonia; environment; pathogen vectors

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1. Introduction

Mosquitoes (Diptera: Culicidae) are common biting insects found on almost every continent: Thus far, a total of 3583 species have been recorded from various parts of the world [1,2]. Moreover, mosquitoes are the primary transmitters, also known as vectors, for many of the most important arthropod-borne diseases [3]. All vector-borne diseases combined account for about 17% of the global disease burden, currently endangering around 80% of the world's population [4]. However, ongoing processes like urbanization, alterations in agricultural practices, deforestation, climate change as well as socioeconomic developments influence the prevalence and geographic ranges of both vectors and vector-borne pathogens [5–7]. Furthermore, different mosquito species can act as the principal vector for the same pathogen depending on whether the transmission cycle takes place in a sylvatic, rural or urban setting [8,9]. Hence, it is not only important to study global mosquito diversity patterns, but to also understand how mosquito communities and vector-human interactions are shaped by local conditions, as mosquitoes are the most likely vectors to cause vector-borne disease epidemics in urban environments [10].

Human habitats present mosquitoes with unique challenges: densely populated areas provide human-biting insects with a reliable food source, giving anthropophilic species a notable evolutionary advantage [11]. Furthermore, cities present a highly fragmented setting, where biodiversity is influenced by the interactions between the microenvironment and urbanization specific broad-scale trends [12–14]. For example, the urban heat island effect is a well-established phenomenon: Large settlements tend to be significantly warmer than the surrounding areas [15–17]. These higher temperatures help create suitable habitats for organisms normally found in lower latitudes, supporting the spread and establishment of invasive species [18,19]. This in turn allows for the northward expansion of exotic vector-borne pathogens, exposing more people to the risk of infections [5,20]. On the other hand, urbanization is most commonly associated with a general decrease in species diversity, affecting specialized organisms more than generalists, although this varies by taxon [12,13,18]. Densely populated settlements naturally contribute to the abundance and development of synanthropic organisms. Therefore, it is to be expected that anthropogenic landscapes favor some mosquito species above others [11,21]. Urban green spaces are particularly noteworthy for providing mosquitoes with ample shelter and a variety of food sources [22,23]. Hence, as mosquitoes can be a severe nuisance as well as present a clear health risk, it is important to develop a better understanding of their community structure in various locations with differing levels of urban development [5,21,24].

Biodiversity, species abundance and the community makeup of anthropogenic landscapes has received increasing attention in the past decades [25]. A number of studies have investigated general mosquito abundance and diversity in various towns and suburban areas as well as how these populations respond to different weather conditions [26–29]. Others have examined the urban lifecycles of the most common or significant synanthropic mosquito species [30,31]. Some studies have concentrated on the ways the characteristics of urban green spaces can influence mosquito abundance, regardless of weather patterns, and how these areas could be designed to be safer for humans [32–34]. Similarly, efforts have been made to improve methods of detecting areas which serve as mosquito refuges and breeding sites [35]. For example, previous research has shown that the container breeding *Culex* (*Cx.*) *pipiens* Linnaeus, 1758 is exceedingly common and abundant in urban environments [26,27]. Nonetheless, in the Po Plain Valley region of Italy it was found that during summer months the overall density of *Cx. pipiens* was still higher in rural sites rather than urban areas [30]. Furthermore, field tests in Thailand indicated that environmental characteristics like closeness of waterbodies and forested areas as well as higher canopy cover increased the number of larvae predators in mosquito breeding sites, but these predators attacked mosquitoes of various species at different rates [34]. Researchers looking at city parks in Manaus, Brazil collected mosquitoes from various distances from the forest edge, revealing a significant difference in the species composition of sites near the perimeter and those 500 m into the forest [36]. On the other hand, a study conducted in Hong Kong

found that while temperature had an overarching effect on urban mosquito populations, windiness had a negative effect on mosquito abundance in rooftop green spaces, making these areas safer for residents than ground level parks [33]. Research in Chicago, IL, USA demonstrated that species richness as well as diversity correlated positively with habitat heterogeneity, and climatic variability appeared to influence mosquito diversity patterns across the study sites [14]. In the same study, doctor Chaves and colleagues also found that an increase in species diversity coincided with a reduction in mosquito abundance. All in all, it is clear that urban mosquito populations are shaped by both largescale progresses as well as the local microhabitat, but the nature and strength of these interactions should be further examined in settlements with various levels of urbanization and in different climate zones [12].

Thus far, most studies regarding urban mosquitoes have been conducted in the tropics, subtropics and the warmer areas of the temperate climate zone. The aim of this study was to better understand the main factors influencing mosquito abundance and species diversity in the urban green spaces of a low density settlement in the boreal biome. For this purpose, four main hypotheses were established:

1. Higher temperature and relative humidity values result in a greater number of active mosquitoes.
2. Stronger winds are expected to have a negative correlation with the number of active individuals.
3. The ratio of collected female and male mosquitoes varies over the warm season, because male mosquitoes have shorter lifespans [37] and thus their abundance should be more sensitive to recent adverse weather conditions.
4. Urban mosquito populations are dominated by one or two abundant synanthropic species.

2. Materials and Methods

This study was conducted in Tartu, the second largest town in Estonia, situated on the east and west shores of river Emajõgi. Weather in Estonia is characterized by the temperate continental climate with cold winters and brief but warm summers according to the Köppen-Trewartha climate classification system [38,39]. The European Commission considers Estonia to belong to the Boreal biome [40]. Tartu itself is a university town with slightly more than 96,000 inhabitants (624.2 inhabitants per km^2) and serves as the regional center for Southern-Estonia [41]. The town area spans 38.80 km^2: This includes 3.90 km^2 (about 10.1%) of urban green spaces and 5.10 km^2 (13.1%) of natural vegetation [42,43].

Mosquitoes were collected using a 50 cm diameter mesh net once a week from May to October during 2013, 2016 and 2017. Hand-net collections have been previously used by numerous researchers [44] and this method was chosen for its cost effectiveness as well as robustness, as it allowed collecting mosquitoes from busy areas where the use of stationary traps was not possible. Collection sites (Figure 1) were visited each week in a changing order starting from five o'clock in the afternoon. All in all, six collection sites were sampled during 2013, one new site was added in 2016 and further eight sites were added in 2017. Collection sites were located in the shaded areas of parks, near play areas, recreational trails or footpaths:

- Site 1 (58°23′40.6″ N, 26°44′05.6″ E) was situated in a corner of an abandoned gravel quarry by a well-traveled park with large trees but very little brush.
- Site 2 (58°23′44.6″ N, 26°43′44.4″ E) was in a sitting area in the town's largest commentary complex, surrounded by both old trees as well as ornamental hedges.
- Site 3 (58°23′24.7″ N, 26°42′55.7″ E) was located on the north shore of river Emajõgi, under sparse old trees.
- Site 4 (58°23′20.1″ N, 26°42′52.6″ E) was situated on the south side of river Emajõgi and included both old park trees as well as brush.
- Site 5 (58°23′05.5″ N, 26°42′19.7″ E) was in Tähtvere park by a large ornamental bush, sparsely surrounded by old trees.

- Site 6 (58°22′17.8″ N, 26°41′58.1″ E) was in Mathieseni park, on the south side of a row of tall ornamental bushes, surrounded by park trees. This park borders the Tartu University Hospital and is visited by both faculty and patients.
- Site 7 (58°22′52.10″ N, 26°42′49.13″ E) was situated on an uneven natural hill called Toomemägi, close to the ruins of a former cathedral. This park is dotted by trees and the irregular features as well as ruined structures offer plenty of shade.
- Site A (58°21′13.4″ N, 26°40′45.5″ E) was located in a tree enclosed green space at the edge of the town.
- Site B (58°21′36.9″ N, 26°41′10.4″ E) was on the border between single-family homes and a small densely wooded area.
- Site C (58°21′1.3″ N, 26°41′30.6″ E) was situated beside a construction site at the edge of the town, with very few trees or bushes in the vicinity.
- Site D (58°21′26.4″ N, 26°42′60.0″ E) was on the margins of Pauluse cemetery, which is dotted by old trees and features a small pond.
- Site E (58°21′50.6″ N, 26°43′43.0″ E) was situated close to the border between the yard of St. Alexander's Orthodox Church and surrounding residential buildings.
- Site F (58°21′36.5″ N, 26°43′56.3″ E) was located in a small parking area surrounded by Forseliuse park, which feature large trees and a children's play area.
- Site G (58°21′23.8″ N, 26°44′31.7″ E) was on a construction site near river Emajõgi, surrounded by large commercial buildings.
- Site H (58°20′52.9″ N, 26°41′37.3″ E) was located in a sparsely populated area near the city limits, overgrown with brush.

Figure 1. Map showing the collection sites in Tartu and the location of Estonia. Sites 1, 2, 3, 4, 5 and 6 were used in 2013, 2016 and 2017. Site 7 was included in the study in 2016 and 2017. Sites A, B, C, D, E, F, G and H were added in 2017. Base map of Tartu: Estonian Land Board (https://xgis.maaamet.ee/xgis2/page/app/maainfo, accessed on 18 November 2020), 2019. Map of Europe: © MapTiler; © OpenStreetMap contributors (https://www.maptiler.com/, accessed on 18 November 2020).

The collection protocol called for two times 25 swings with the insect net and specimens were gathered between as well as after the sets with an aspirator. Net swings were made in the air and through the tips of soft vegetation. Date, time and the person collecting mosquitoes was recorded at each site. Mosquitoes were later killed by freezing and stored in 75% ethanol (C_2H_5OH) or as dry material at −20 °C. Specimens were identified to species or a species group level under a stereomicroscope Olympus SZ61 (Olympus Corporation, Shinjuku, Tokyo, Japan) using a standard taxonomic key [37] and their gender was recorded. Mosquitoes too damaged for identification were marked as "unspecified". Afterwards, weather information was added to the data from the records of the Estonian Weather Service, based on the date and time of fieldwork. Data was acquired from Tartu-Tõravere meteorological station (58°15′51″ N, 26°27′41″ E), which is situated about 16 km southwest (SW) of the city limits of Tartu. Each catch in the dataset was provided with the measurements of time to sundown (min), temperature (°C), relative humidity (%), wind speed (m/s) and atmospheric pressure at sea level (hPa).

Shannon diversity indices (H) were calculated based on the number of mosquitoes and the quantity of different species in each catch [45]. The Shannon diversity index can be written as the following equation:

$$H = -\sum_{i=1}^{S} p_i \ln p_i$$

where S is the number of different mosquito species and p is the number of individuals of the same species divided by the number of all individuals. From this, the true diversity of the collected mosquito samples was calculated using the effective number of species (*ENS*). This statistic indicates what kind of a population with equally represented species the examined sample is similar to [46]. *ENS* was calculated by taking the exponential of the Shannon diversity index and the results were rounded to integers:

$$ENS = \exp\left(-\sum_{i=1}^{S} p_i \ln p_i\right).$$

As the mosquito count data had a Poisson distribution, the parameter lambda (λ) was used to represent the average number of mosquitoes caught during collection events. For the same reasons, 95% confidence interval (CI), instead of standard deviation, was used to characterize dispersion. Additional statistical analyses were done in the free software R version 3.6.1 [47]. Mosquitoes which could not be identified to species or species group level were only included in the dataset when analyzing specimen yields and removed when examining species diversity. Additionally, data was cleaned of outliers and the independent variables were checked for pairwise correlations using R package "psych" [48]. The degree of correlation was evaluated using the non-parametric Kendall rank correlation coefficient (τ). As relative humidity and time until sunset were moderately correlated with each other ($\tau = -0.43$) as well as the month ($\tau = 0.45$ and $\tau = -0.44$, respectively) they were dropped from the analysis. As no fieldwork was done in October in 2013 and only few catches were made during that month in 2016, the records for October were also eliminated from the dataset. Days when fieldwork was terminated early due to rainfall were removed.

Using the R package "MASS" [49], negative binomial generalized linear model (GLM) was employed to determine the character and power of the relationship between the independent variables (collection site, month and year of collection, temperature, wind speed, relative humidity and gender) and the number of collected mosquitoes. This was due to the dataset exhibiting both over dispersion and zero inflation. On the other hand, a GLM with Poisson distribution was used for modeling the relationships between independent variables and ENS. Non-significant variables were removed from the models by hand. Models were tested for over- and under-dispersion as well as zero inflation using the R packages "DHARMa" [50] and "performance" [51], respectively. Furthermore, the R package "mctest" [52,53] was employed to evaluate the level of multicollinearity

among the independent variables based on variance inflation factor (VIF) and tolerance (TOL). Illustrative figures were generated using the R package "ggplot2" [54]. When necessary, correlation statistics included on these figures were calculated by conducting a non-parametric test using Kendall rank correlation.

3. Results

The dataset analyzed in this study consisted of 1890 mosquitoes caught from 15 collection sites in the town of Tartu: 654 mosquitoes were collected in 2013 (74.01% of these were female), 556 in 2016 (53.60% female) and 680 in 2017 (58.97% female). Of these individuals, 47 mosquitoes were too damaged to be identified by their morphological traits. It should be stressed, that in 2013 six collection sites were sampled, in 2016 one new site was added and in 2017 a total of eight additional sites were added. Therefore, while the total number of collected mosquitoes was similar between the three years, in reality the mean number of individuals caught during each collection event decreased from 6.41, 95% CI [6.22–6.61] (Poisson lambda (λ), 95% confidence interval (CI) [lower limit–upper limit]) in 2013 to 3.78, 95% CI [3.62–3.94] in 2016 and 2.53, 95% CI [2.41–2.65] in 2017. The number of mosquitoes caught during one collection event varied from zero to 90 and was influenced by year, month, temperature, wind conditions, insect gender as well as study site, but also by the associations between these factors (Table 1). Species diversity, represented by the effective number of species (ENS), also showed a slight decrease between the three years: the average ENS was 1.59, 95% CI [1.40–1.78] in 2013, 1.39 [1.23–1.55] in 2016 and 1.11 [0.99–1.23] in 2017. Additionally, the ENS of a single collection event only varied from zero to six and was influenced by the collection year and site (Table 2).

The number of collected mosquitoes was dependent on the collection year and month as well as on the interaction between the two variables (Figure 2). On average, mosquito collection events yielded far more individuals during 2013 than during 2016 and 2017. However, there was also marked variance between the fieldwork months. All in all, higher numbers of mosquitoes were caught during May and June. Noticeably fewer mosquitoes were collected on average during July, August and September. Interestingly, when looking at how the interactions between year and month influence average mosquito yield, it seems that in 2016 and 2017 the average number of mosquitoes collected during May is significantly smaller than in 2013 compared to the other months. Because of this, the interactions between the later years and other collection months, except for June in 2016, show a positive effect on the average mosquito yield.

Somewhat surprisingly, higher temperatures appeared to correlate with fewer collected mosquitoes (Figure 3). Interestingly, there was a negative association between temperature and relative humidity (Figure 4). On the other hand, as could be expected, stronger winds in the area of the town resulted in fewer mosquitoes being collected during fieldwork. However, there was no significant interaction between individual study sites and general wind conditions. Quite predictably, male mosquitoes were collected much less often than females. Additionally, there appears to be an interaction between collection month and insect gender. The proportion of males among the collected mosquitoes was overall significantly larger in August than in May. This difference becomes even more pronounced in September. However, there was no significant interaction between collection year and gender. Furthermore, some of the 15 study sites yielded more mosquitoes on average than others.

The effective number of species (ENS) statistic was chosen to represent population diversity. ENS was calculated for every collection event, based on all of the mosquitoes that could be identified to species or species group level by morphological markers. Results show that the diversity of the collected individuals was influenced by both collection site and year (Figure 5). ENS did not appear to be influenced by the study month, temperature, wind conditions or atmospheric pressure. Furthermore, some collection sites yield more mosquito species on average than the reference site. As with mosquito abundance, the

average effective number of species decreased from 2013 to 2017. Interestingly, the overall number of recorded species actually increased during the study.

Table 1. Generalized linear model (GLM) results showing how independent variables influence the number of collected mosquitoes.

Explanatory Variables	**β**	**±SE**	**Cl 2.5%**	**Cl 97.5%**	**z Value**	***p* Value**
(Intercept)	3.526	0.561	2.40	4.63	6.290	<0.001 ***
Temperature	−0.099	0.019	−0.138	−0.060	−5.210	<0.001 ***
Wind conditions	−0.129	0.065	−0.262	0.005	−1.991	0.047 *
Study Site (Ref: Site A)						
Site B	0.852	0.481	−0.077	1.817	1.771	0.077
Site C	1.080	0.478	0.168	2.032	2.261	0.024 *
Site D	1.465	0.460	0.601	2.380	3.185	0.001 **
Site E	2.092	0.447	1.244	2.996	4.678	<0.001 ***
Site F	2.789	0.439	1.957	3.680	6.361	<0.001 ***
Site G	1.637	0.458	0.773	2.553	3.573	<0.001 ***
Site H	2.762	0.442	1.928	3.654	6.252	<0.001 ***
Site 1	1.440	0.415	0.663	2.272	3.465	0.001 ***
Site 2	0.070	0.429	−0.739	0.928	0.163	0.871
Site 3	0.197	0.428	−0.609	1.053	0.460	0.645
Site 4	0.731	0.421	−0.064	1.578	1.737	0.082
Site 5	0.534	0.423	−0.267	1.386	1.262	0.207
Site 6	1.231	0.416	0.446	2.070	2.956	0.003 **
Site 7	0.806	0.431	−0.003	1.666	1.871	0.061
Collection Year (Ref: 2013)						
2016	−1.948	0.317	−2.617	−1.287	−6.139	<0.001 ***
2017	−3.627	0.331	−4.315	−2.962	−10.965	<0.001 ***
Collection Month (Ref: May)						
June	−0.479	0.353	−1.117	0.159	−1.357	0.175
July	−1.844	0.339	−2.492	−1.206	−5.444	<0.001 ***
August	−1.584	0.334	−2.234	−0.943	−4.747	<0.001 ***
September	−3.558	0.409	−4.366	−2.764	−8.710	<0.001 ***
Gender (Ref: Female)						
Male	−0.887	0.264	−1.435	−0.335	−3.364	<0.001 ***
Interactions between Year (Ref: 2013) and Month (Ref: May)						
2016: June	0.675	0.440	−0.217	1.564	1.533	0.125
2017: June	1.639	0.415	0.823	2.458	3.946	<0.001 ***
2016: July	2.167	0.427	1.285	3.052	5.069	<0.001 ***
2017: July	2.743	0.413	1.909	3.586	6.642	<0.001 ***
2016: August	1.601	0.414	0.750	2.453	3.864	<0.001 ***
2017: August	1.673	0.408	0.861	2.491	4.100	<0.001 ***
2016: September	3.390	0.457	2.457	4.329	7.420	<0.001 ***
2017: September	4.026	0.466	3.104	4.959	8.634	<0.001 ***
Interactions between Month (Ref: May) and Insect Gender (Ref: Female)						
June: Male gender	−0.175	0.341	−0.872	0.518	−0.515	0.607
July: Male gender	0.229	0.333	−0.452	0.906	0.686	0.493
August: Male gender	0.817	0.333	0.135	1.496	2.452	0.014 *
September: Male gender	1.197	0.333	0.507	1.884	3.594	<0.001 ***

Deviance residuals: min = −2.1444; 1Q = −1.0263; median = −0.6284; 3Q = 0.2327; max = 3.2837. Theta: 0.7169, standard error (SD): 0.0575. Null deviance 1410.84 on 1035 degrees of freedom (df), residual deviance 945.81 on 1000 df. Significance symbols: 0.05 to 0.01 = "*", 0.01 to 0.001 = "**", <0.001 = "***". Abbreviation as follows: Estimates (β), standard error (±SE) and confidence limit (Cl).

Table 2. Generalized linear model (GLM) results showing how collection site and year influenced the effective number of species (ENS).

Explanatory Variables	β	±SE	CI 2.5%	CI 97.5%	t Value	*p* Value
(Intercept)	−0.065	0.357	−0.835	0.58	−0.182	0.856
Collection Sites (Ref: Site A)						
Site B	0.442	0.427	−0.382	1.318	1.034	0.301
Site C	0.747	0.405	−0.019	1.589	1.847	0.065
Site D	0.747	0.405	−0.019	1.589	1.847	0.065
Site E	1.269	0.377	0.571	2.069	3.362	<0.001 ***
Site F	1.541	0.367	0.868	2.326	4.194	<0.001 ***
Site G	0.636	0.412	−0.149	1.489	1.543	0.123
Site H	1.598	0.367	0.925	2.383	4.350	<0.001 ***
Site 1	0.893	0.358	0.243	1.665	2.491	0.013 *
Site 2	0.343	0.368	−0.330	1.130	0.931	0.352
Site 3	0.180	0.372	−0.502	0.974	0.484	0.628
Site 4	0.553	0.364	−0.110	1.333	1.519	0.129
Site 5	0.256	0.370	−0.422	1.046	0.691	0.490
Site 6	0.737	0.361	0.082	1.513	2.044	0.041 *
Site 7	0.328	0.380	−0.374	1.134	0.863	0.388
Study Years (Ref: 2013)						
2016	−0.109	0.107	−0.319	0.102	−1.013	0.311
2017	−0.628	0.127	−0.881	−0.381	−4.932	<0.001 ***

Deviance residuals: min = −2.2229; 1Q = −1.1782; median = −0.1585; 3Q = 0.6215; max = 2.9675. Null deviance 706.51 on 517 degrees of freedom (df), residual deviance 595.01 on 501 df. Significance symbols: 0.05 to 0.01 = "*", <0.001 = "***". Abbreviation as follows: Estimates (β), standard error (±SE) and confidence limit (CI).

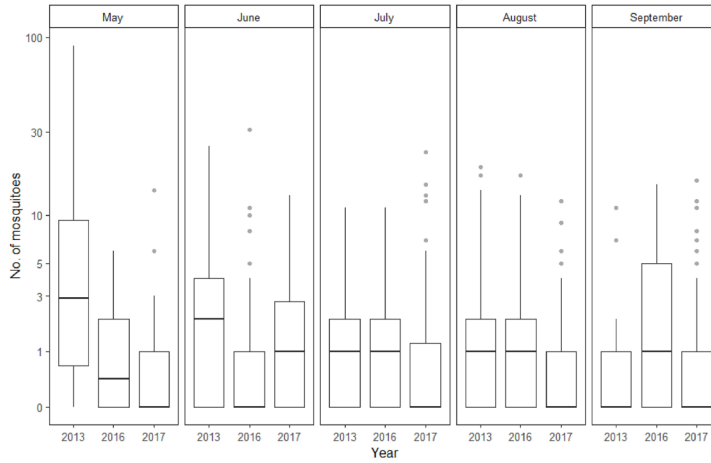

Figure 2. Average number of mosquitoes collected during the different months of the study period. Boxplots showing the median (dark line dividing the box), interquartile range (IQR) containing 50% of the data points (length of the box), upper and lower quartiles (whiskers) and outliers (gray dots). *Y*-axis has been transformed to a logarithmic scale for ease of viewing.

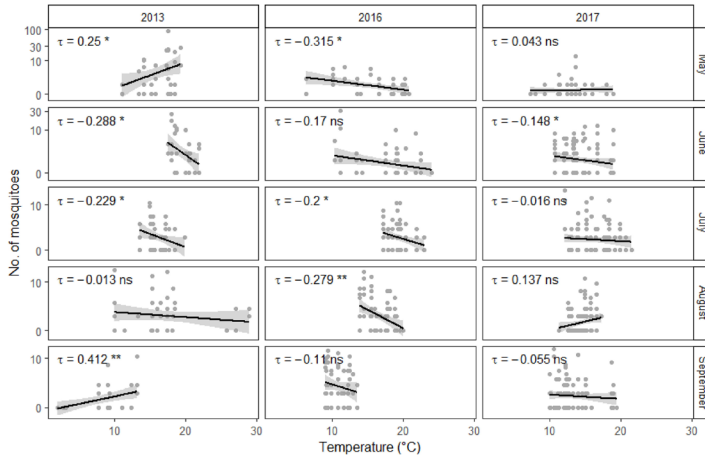

Figure 3. Influence of temperature on the abundance of mosquitoes during different months. Y-axis has been transformed to a logarithmic scale for ease of viewing. Gray points represent fieldwork results. Linear regression lines are surrounded by gray areas representing 95% confidence intervals. Correlation statistics have been calculated using the non-parametric Kendall rank correlation. Significance: >0.05 = "ns", 0.05 to 0.01 = "*", 0.01 to 0.001 = "**".

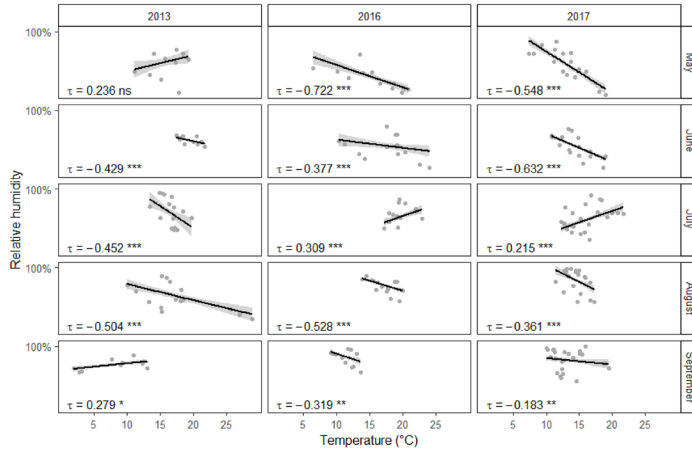

Figure 4. Correlation between temperature and relative humidity. On many occasions higher temperatures correlated with lower relative humidity. Collection events are represented by gray dots, linear regression lines are surrounded by gray areas representing 95% confidence intervals. Correlation statistics have been calculated using the non-parametric Kendall rank correlation. Significance: >0.05 = "ns" 0.05 to 0.01 = "*", 0.01 to 0.001 = "**", <0.001 = "***".

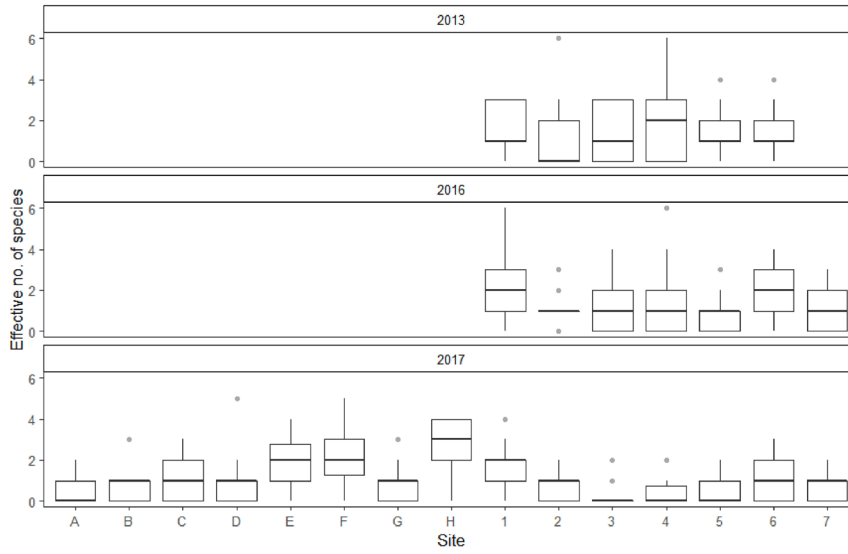

Figure 5. Average effective no. of species (ENS) of each collection site throughout the three study years. Boxplots showing the median (dark line dividing the box), interquartile range (IQR) containing 50% of the data points (length of the box), upper and lower quartiles (whiskers) and outliers (gray dots).

All in all, 20 different mosquito species and species groups from five genera (*Aedes* Meigen, 1818, *Anopheles* Meigen, 1818, *Coquillettidia* Dyar, 1904, *Culex* Linnaeus, 1758 and *Culiseta* Felt, 1904) were collected during the study period—14 species in 2013 and 17 in both 2016 as well as 2017. There are thought to be about 32 mosquito species in Estonia [55]. *Culex* (*Culex*) *pipiens* Linnaeus, 1758 together with *Cx.* (*Culex*) *torrentium* Martini, 1925 were the most collected mosquitoes during all three study years. However, the abundance of other common species varied dramatically from year to year (Table 3). Furthermore, the dominant species changed within each study year as the warm season progressed (Figure 6). In 2013, the species or species group most commonly collected in May was *Ae. communis* (de Geer, 1776), and in June *Ae. annulipes* group. *Cx. pipiens*/*Cx. torrentium* were dominant throughout the remaining warm season. On the other hand, the years 2016 and 2017 were similar to each other. In both years *Ae.* (*Ochlerotatus*) *punctor* (Kirby, 1837) together with *Ae.* (*Ochlerotatus*) *punctodes* (Dyar, 1922) and *Ae. annulipes* group dominated in May and June, respectively. *Cx. pipiens*/*Cx. torrentium* group as well as the *Ae. cinereus*/*Ae. geminus* group were most numerous in July and August. As expected, *Cx. pipiens*/*Cx. torrentium* were the predominant individuals in September.

Table 3. List of mosquito species and groups collected during the study in alphabetical order. The table contains the number of individuals from each identified taxon, followed by the percentage (%) of female mosquitoes. Six collection sites were sampled during 2013, seven sites in 2016 and 15 collection points in 2017. The mean number of mosquitoes caught during a collection event was 6.41 in 2013, 3.78 in 2016 and 2.53 in 2017.

Species	2013 Total	% Female	2016 Total	% Female	2017 Total	% Female
Unspecified	15	93.33	21	85.71	11	90.91
Aedes (Aedes) cinereus geminus	45	66.67	109	49.54	108	57.41
Aedes (Aedimorphus) vexans (Meigen, 1830)	4	75.00	40	67.50	31	64.52
Aedes (Ochlerotatus) annulipes group	106	94.34	74	48.65	133	75.19
Aedes (Ochlerotatus) cataphylla Dyar, 1916	0	NA	7	85.71	29	86.21
Aedes (Ochlerotatus) communis (de Geer, 1776)	138	95.65	19	42.11	23	73.91
Aedes (Ochlerotatus) diantaeus Howard, Dyar and Knab, 1913	1	0.00	0	NA	0	NA
Aedes (Ochlerotatus) excrucians (Walker, 1856)	16	81.25	6	66.67	8	75.00
Aedes (Ochlerotatus) flavescens (Müller, 1764)	1	100.00	2	50.00	0	NA
Aedes (Ochlerotatus) intrudens Dyar, 1919	106	95.28	3	100.00	2	100.00
Aedes (Ochlerotatus) leucomelas (Meigen, 1804)	0	NA	2	50.00	7	85.71
Aedes (Ochlerotatus) pullatus (Coquillett, 1904)	0	NA	1	100.00	0	NA
Aedes (Ochlerotatus) punctor/punctodes	44	75.00	38	52.63	76	67.11
Aedes (Ochlerotatus) sticticus (Meigen, 1838)	0	NA	36	94.44	26	84.62
Anopheles (Anopheles) claviger (Meigen, 1804)	3	0.00	0	NA	1	100.00
Anopheles (Anopheles) maculipennis complex	4	25.00	3	33.33	2	100.00
Coquillettidia (Coquillettidia) richiardii (Ficalbi, 1889)	11	72.73	2	100.00	10	80.00
Culex (Culex) pipiens/torrentium	151	30.46	163	43.56	202	33.17
Culex (Neoculex) territans Walker, 1856	9	22.22	27	33.33	4	0.00
Culiseta (Culicella) ochroptera (Peus, 1935)	0	NA	3	66.67	1	100.00
Culiseta (Culiseta) annulata (Schrank, 1776)	0	NA	0	NA	6	16.67
Total	654		556		680	

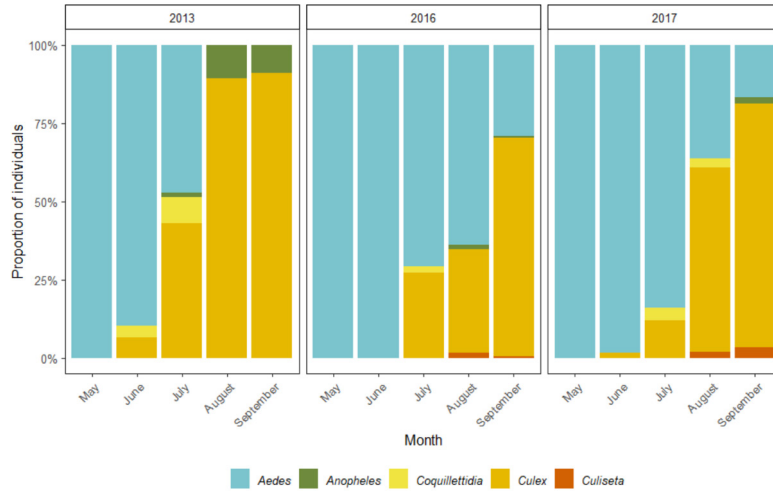

Figure 6. The succession of mosquitoes from different genera over the study period. The percentage of mosquitoes from five different genera collected in 2013, 2016 and 2017, showing the transition from *Aedes* to *Culex* dominated populations during the warm months.

4. Discussion

The chosen collection method only allowed to capture a relatively small sample, 1890 individuals, of the local mosquito population, but this was sufficient to illustrate how different environmental factors influence the quantity and diversity of active mosquitoes in urban green spaces. Results show that both the mean number of collected mosquitoes as well as the average effective number of species decreased significantly from 2013 to 2017, while the number of recorded species actually increased. There have been no coordinated mosquito control efforts in Tartu, therefore the differences in abundance and variety are probably due to changes in annual weather patterns. Furthermore, it is likely that the town environment further amplified the effects of some of these atmospheric conditions.

Temperature, precipitation and humidity are considered to be the most important weather factors influencing mosquito abundance [5]. Additionally, these aspects are not only important during the warm season: weather conditions during winter and early spring can also have a profound effect on bloodsucking insects. For example, many of the spring mosquito species rely on snowmelt pools or flood waters for the development of the new generation and therefore require snowy winters [37]. In fact, this is the most likely explanation for why fieldwork in May 2013 yielded so many more mosquitoes on average compared to 2016 and 2017. According to the Estonian Weather Service, snow could be found everywhere in Estonia during the first three months of 2013, and the snow cover finally completely disappeared by the end of April, resulting in routine flooding [56]. On the other hand, snow conditions were less stable during the first months of 2016, with the snow partially melting many times during January, February and March, then finally disappearing at the beginning of April [57]. January and February of 2017 were especially warm and snow could only form thin layers on the ground, similar pattern continued in March with the snow cover melting and reforming many times until the end of the month [58]. Due to the absence of snow at the end of April during the last two study years, there was likely less floodwater available for the mosquito larvae that depend on it. Moreover, the mean temperature and the amount of rainfall in May also differed substantially from 2013 to 2017. May in 2013 was warm (mean temperature 2.9 °C higher than normal) as well as rainy (mean precipitation 22 mm higher than normal), while 2016 was warm (mean temperature 2.7 °C higher than normal) but dry (mean precipitation 24 mm less than normal) and 2017 was cool (mean temperature 1.1 °C colder compared to normal) as well as dry (mean precipitation 27 mm less than normal) [56–58]. Both winter snow cover as well as weather conditions in May likely played a significant role in the decrease of spring and early summer mosquitoes from 2013 to 2017. Furthermore, it has been previously reported, that the mean relative humidity in May can strongly influence the insect abundance throughout the rest of the warm season [30]. It is clear that mosquitoes started off with high abundance in 2013, but the number of individuals noticeably decreased over the rest of the warm season. However, the number of mosquitoes caught during collection events followed the complete opposite trajectory in 2016, when September yielded the most specimens. Furthermore, the 2017 study year proved the most variable as the number of collected mosquitoes was similarly low in May but also noticeably dropped in August. All this indicates that the variations in local weather conditions between years and months play an important role in the number of actively flying mosquitoes.

Undoubtedly, air temperature is an important factor in determining the development speed of mosquito larvae and air temperature often correlated with the number of mosquitoes collected during the three years of this study. However, warmer temperatures were somewhat surprisingly often associated with fewer captured mosquitoes. This could be explained by the negative correlation between temperature and relative humidity. Mosquitoes are relatively delicate insects and risk drying out in direct sunlight and low humidity conditions. Therefore it is not surprising that high relative humidity is positively correlated with higher numbers of active mosquitoes [59]. At the same time, urban environments have been shown to have lower relative humidity and higher temperature values

than the surrounding areas [17,60]. This most likely means that the collection sites were in reality even warmer and drier than what the closest weather station measured. However, there were also exceptions to the general trend, when the relationship between temperature and the number of collected mosquitoes could not be explained by the level of relative humidity. Regrettably, this study fails to offer an alternative explanation to these cases. Finally, there was also a statistically important positive correlation between temperature and the number of active mosquitoes during September 2013, likely because temperatures had dropped below +5 °C, which had a noticeable negative impact on collection success. All of this taken together means that the first hypotheses postulated in this study is not completely correct. While both higher temperature and humidity values are favorable for mosquitoes, there can be a negative correlation between the two factors.

Wind conditions were also shown to affect mosquitoes and the second hypotheses of this study was proven correct. Stronger winds had a predictably negative correlation with the average number of individuals collected during fieldwork. Although moderate wind speeds can be helpful to mosquitoes by facilitating long distance dispersal and by carrying host scent further down-wind, many mosquito species have trouble flying in windy conditions [37,61,62]. For example, strong winds have been proposed to be the main reason why mosquitoes avoid inhabiting urban green roofs [33]. It should be noted that there was no significant interaction between wind speed and collection site in the current study, indicating that none of the sites were more protected from the wind than others. All in all, it could be advantageous to take wind conditions into consideration when deigning urban green spaces, in order to avoid creating areas which could become too shielded from the wind and facilitate mosquito biting activity.

Collecting mosquitoes with an insect net made it possible to capture both female and male individuals. Although only female mosquitoes require blood and thus act as disease vectors and pests, a better understanding of the male population is also necessary for the development of effective mosquito control measures [63]. Predictably, female mosquitoes were collected more often than males: Although many species exhibit a 1:1 sex ratio and some are even male biased [64], female mosquitoes were likely attracted to the person conducting fieldwork, while males were caught more randomly. However, there was also an interaction between mosquito gender and month. More male mosquitoes were collected in the last two months of the study period compared to May and this change was not paralleled by females. This could be explained in part because *Cx. pipiens* females, one of the most numerous species in August and September, do not usually take a blood meal before overwintering [37] and thus were less likely to be drawn to the fieldworker. On the other hand, it seems that yearly weather changes influence both genders similarly as there was no statistically significant interaction between mosquito gender and collection year. All in all, hypotheses number three of this study was not proven correct. Although the sex ratio of the collected mosquitoes varied over the fieldwork period, these differences were better explained by other factors than weather fluctuations during the warm season.

Some collection sites yielded significantly more mosquitoes on average than the reference site A. Out of the six spots that were visited during all of the collection years, more mosquitoes were caught at sites 1 and 6. Yet, this result cannot be explained by the factors accounted for in this study. There was no discernable interaction between collection sites and wind. Neither do sites 1 and 6 noticeably differ from the others in the availability of mosquito breeding sites. Furthermore, sites C, D, E, F, G and H also yielded significantly more mosquitoes on average compared to the reference site. However, care should be taken when comparing sites A through H to sites 1 to 7, as these were collected from by different people and collector bias cannot be excluded. Finally, there are also landscape factors that can influence mosquito abundance and help explain the variation between collection sites [32], but these are outside the scope of this study.

The average effective number of species (ENS) was only significantly different between 2013 and 2017, with the fieldwork results of the later year displaying less species diversity. This was most likely due to the 2017 study year yielding fewer collected mosquitoes in

general and normally rare species were even less likely to be caught. This was further reflected in fact that the collection sites E, F, H, 1 and 6, which had some of the best mosquito yields on average, were also the ones with the highest mean ENS results. Although, there were some differences: sites G and D did not appear to have a statistically significant effect on ENS, but exhibited a larger positive effect on the average number of collected mosquitoes than sites 1 and 6. Hence, there likely is some variation in species diversity between the collection sites that cannot be explained by a larger number of sampled individuals.

Species succession throughout the warm season follows predictable patterns. *Ae. communis*, *Ae. intrudens* and *Ae. punctor*/*Ae. punctodes* are snowmelt mosquitoes able to tolerate colder conditions [37] and thus were understandably most numerous in May. Interestingly, while *Ae. communis* and *Ae. intrudens* was exceedingly numerous in 2013, they were much less prominent in the later study years. *Ae. intrudens* in particular was almost absent in 2017. While the number of collected *Ae. punctor*/*Ae. punctodes* individuals also fell during 2016 and 2017, the change was much less dramatic. Mosquitoes from the *Ae. annulipes* group and *Ae. cinereus*/*Ae. geminus* also appeared during the beginning of the warm season, overtaking snowmelt mosquitoes as the most numerous species in June and July, with some variation between the years. Individuals of the *Ae. annulipes* group could be found from May to August but were most numerous in June. However, in 2013 the largest number of *Ae. cinereus*/*Ae. geminus* mosquitoes were collected in May, but no individuals from these species could be found in August or September. On the other hand, in the later study years *Ae. cinereus*/*Ae. geminus* were much more numerous during the summer months and could still be collected throughout September. *Ae. cinereus* and *Ae. geminus* are thought to prefer semi-permanent water features, but also require warmer temperatures than the snowmelt mosquitoes [37]. Therefore, May being both warm and rainy in 2013 most likely explains why these species were active early in the season at the time, but suitable breeding sites likely dried up over the summer. *Cx. pipiens* and *Cx. torrentium* were the most enduringly abundant species during the study period: every year first individuals started appearing at the beginning of summer and became dominant in September. Other various species were caught in low numbers, mostly over the summer months. All in all, it appears that the fourth hypothesis of this study was proven correct: the synanthropic species *Cx. pipiens* was abundant during every year of the study, while a few other species were dominant in some years but not others. Additionally, the 20 species collected in this study constitute about 62.5% of the overall mosquito richness in Estonia. Compared to the Estonian checklist, which was compiled in 1955 [65] and updated in 2014 [55], the urban mosquito fauna of Tartu is missing halophilic species as well as some species from the genera Culiseta. Some of the rarer *Aedes* species of Estonia were also not collected during this study. Still, the relatively high level of collected species indicates that the urban green spaces of Tartu encompass various microhabitats able to support both synanthropic and sylvan mosquitoes. Such environmental variety is commendable from a general biodiversity perspective but may also imply that it is more likely for future invasive mosquito species to become locally established.

Furthermore, the species most numerous in Tartu are all known disease vectors. For example, prior studies have identified *Cx. pipiens* and/or *Cx. torrentium* individuals infected with the West Nile virus [66,67], Ockelbo virus [68], Usutu virus [69], *Borrelia* (*B.*) *garinii* [70], *Francisella* (*F.*) *tularensis* [71], *Dirofilaria* (*D.*) *repens* and *D. immitis* [72,73]. Moreover, *Cx. pipiens* s.l. can play a key role in transferring pathogens between birds and humans [74]. *Ae. cinereus* mosquitoes have been associated with the Jamestown Canyon virus [75], Ockelbo virus [68], both *B. afzelii* and *B. garinii* [70], *F. tularensis* [71] as well as *D. repens* and *D. immitis* [76,77]. Lastly, the different species of the *Ae. annulipes* group have also been previously indicated in the transmission of *F. tularensis* [71] and *D. repens* [77].

Future work could sample both urban and rural habitats for comparison. Furthermore, establishing collection points in private yards would permit the use of stationary passive insect traps without losing the ability to collect male mosquitoes. These measures would

give a more granular overview of how various conditions influence the changes in mosquito abundance and diversity.

5. Conclusions

The numbers of active mosquitoes inhabiting urban green spaces in the town of Tartu are greatly influenced by the variations in yearly weather patterns. The mean number of collected mosquitoes sharply declined between 2013 and 2017. This could have been in large part because of the winter snow conditions and the meteorological character of the end of spring, as May 2013 was set apart of the other study years by an abundance of snowmelt water as well as a warm and rainy weather. The number of active mosquitoes was also influenced by temperature, humidity and wind. Importantly, there was an apparent negative correlation between temperature and humidity, something that the urban environment most likely further enforced. This means that higher temperatures in the urban environments of the boreal biome may in some cases actually result in fewer active mosquitoes. Furthermore, stronger winds also decreased the number of collected mosquitoes. This is something that could be taken into account when planning for new urban parks with less mosquito biting activity. On the other hand, the diversity index of the collected mosquitoes, represented by the effective number of species (ENS), also declined from 2013 to 2017. This was most likely in part a side effect of the general degrease in the mean number of collected mosquitoes. All in all, *Cx. pipiens* together with *Cx. torrentium* remained the most abundant mosquito species throughout the three study years. Other dominant species tended to vary between the years. Worryingly, the most numerous species collected in this study are all capable of carrying several pathogens. In the light of ongoing anthropogenically driven environmental changes, the surveillance of mosquitoes as well as vector-borne pathogens is becoming increasingly necessary in colder climate zones.

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Rediscovering the mosquito fauna (Diptera: Culicidae) of Estonia: an annotated checklist with distribution maps and DNA evidence

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Abstract

Female mosquitoes (Diptera: Culicidae) affect their hosts in numerous negative ways and are crucial to the spread of vector-borne pathogens. It is, therefore, important to have a detailed overview of regional mosquitoes, to be able to detect changes in species diversity and identify possible health threats. The aim of this study was to update the checklist of the mosquito fauna of Estonia for the first time since 1957. For this purpose, 24,344 adult mosquitoes (94% females) were collected in Estonia from 2008 to 2020 using various trapping methods. Specimens were primarily identified by morphological characteristics, but DNA barcoding based on the partial cytochrome c oxidase subunit 1 gene (*COI*) was also used. Species were included in the checklist based on historical records as well as new collections, while also considering reports from neighboring countries. Species records are supplemented with voucher specimens, distribution maps and DNA evidence. The updated checklist includes 34 species, 27 of which were confirmed with recently collected material. All in all, *Aedes communis* (de Geer, 1776) proved to be the most common mosquito in Estonia, accounting for 30.1% of the specimens collected. This is noteworthy, as this species has been implicated in the transmission of multiple disease agents present in the area. New evidence revealed the presence of *Ae. hexodontus* Dyar, 1916, *Ae. sticticus* (Meigen, 1838), *Anopheles messeae* Falleroni, 1926 and *Culiseta bergrothi* (Edwards, 1921) in Estonia.

Key words: *Aedes*, *Anopheles*, *Coquillettidia*, *Culex*, *Culiseta*, DNA barcoding, entomology, Estonia

Introduction

Mosquitoes (Diptera: Culicidae) are a notable group of insects, as they can affect the wellbeing of humans and animals alike. The haematophagous females of many mosquito species can be a serious biting nuisance, as well as transmit various pathogens. Illnesses caused by vector-borne pathogens affect more than one billion people per year, with diseases caused by mosquito-borne pathogens being responsible for the majority of the infections (WHO 2014). Furthermore, these diseases cause not only death and disability, but also notable monetary loss, further exacerbating economic inequality, as poorer populations are more vulnerable to insect bites (WHO 2017). Blood seeking mosquitoes can also be a nuisance in their own right, disrupting outdoor activities and creating considerable stress in humans and animals (Islam *et al.* 2017). For these reasons, mosquitoes continue to be an important subject of study, as better understanding of their biology and ecology can help predict changes and create strategies to mitigate some of the harmful effects. Mosquito species richness varies based on geographic location, with areas close to the equator supporting a greater number of species than regions at higher latitudes (Foley *et al.* 2007). This diversity makes mosquitoes especially significant in the tropics. However, some important species, a number of which are known to be competent vectors of pathogenic agents, can be found in colder climates as well (Martinet *et al.* 2019). This trend is mirrored by mosquito-borne pathogens: while the majority are confined to warm climates, a number of diseases also occur in higher latitudes and many infections are now emerging or re-emerging (Bale 2012; Liang

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et al. 2015; Evans & Peterson 2019). In fact, throughout recent decades, there have been noticeable changes in the geographic distributions of both biting insects as well as vector-borne pathogens (Medlock *et al.* 2012; Brugueras *et al.* 2020). Such shifts have been driven by numerous anthropogenic and environmental factors, such as global transport routes, changes in land use, urbanization, extreme weather events and climatic fluctuations, among others (Hui 2006; Zell *et al.* 2008). These aspects can also cause significant changes in the relative and absolute abundance of indigenous mosquito species (Franklinos *et al.* 2019; Câmara *et al.* 2020). As a result, calls have been made for increased mosquito surveillance as well as additional empirical studies to investigate vector ecology in the changing world (Franklinos *et al.* 2019).

More than half of the eight Nordic-Baltic countries, as well as the neighboring Russian Federation, have published at least one update to their mosquito checklists during the last few decades. For example, a literature based list of Lithuanian Diptera was published in 2000 and included 36 mosquito species, listing five species in the genus *Anopheles* Meigen, 1818, 23 in genus *Aedes* Meigen, 1818, three in *Culex* Linnaeus, 1758, four in *Culiseta* Felt, 1904 and the species *Coquillettidia richiardii* (Ficalbi, 1889) (Pakalniškis *et al.* 2000). Eleven years later, the official number of Lithuanian mosquitoes rose to 37, with the addition of *Aedes geminus* Peus, 1970 (Bernotienė & Lučiūnaitė 2011). Meanwhile, only 25 mosquito species had been reported from Latvia: four species of *Anopheles*, 17 species of *Aedes*, one species of *Coquillettidia* Dyar, 1905 and *Culex*, as well as two species of the genus *Culiseta*, as reported by Spungis (2000). However, the author of the aforementioned study concluded that the real number of mosquitoes in Latvia was likely to be significantly higher. During this time, the mosquito checklist for European Russia was also revised, with the update featuring 64 species, including four species with doubtful presence (Gornostaeva 2000). The Swedish mosquito fauna has been relatively well researched from 2000 onwards. The most recent checklist for Sweden, based on both prior literature records and new collection efforts (Lundström *et al.* 2013), included 49 mosquito species: seven belonging to the genus *Anopheles*, 31 to *Aedes*, one to *Coquillettidia*, three to *Culex* and another seven to *Culiseta*. At the moment, 55 mosquito species are thought to be present in Sweden (Möhlmann *et al*., 2017; Robert *et al.* 2019). The mosquito fauna of Finland has been updated multiple times in the last decade. First of these was a literature review listing 38 mosquito species (Huldén & Huldén 2014), but this information was further built upon and corrected in later articles (Culverwell 2018; Culverwell *et al.* 2020; Culverwell *et al*. 2021), with the most recent list including 43 species. Similarly, a recent comprehensive overview was written about the mosquitoes of northwestern Russia, reporting a total of 46 species and comparing the results to data from neighboring countries (Khalin & Aibulatov 2020). This was followed by a publication about the northernmost records of these mosquito species (Khalin & Aibulatov 2021). In contrast, the most recent checklist of the mosquitoes of Estonia was published in the mid-Twentieth Century (Remm 1957).

Few people have studied mosquitoes in the area of present-day Estonia. Some of the earliest records concerning the Baltic mosquito fauna can be found from the first half of the Nineteenth Century onwards, attributable to the Baltic German entomologists B. A. Gimmerthal (1779–1848) and F. L. F. Sintenis (1835–1911), as well as some visiting scientists, e.g. A. Dampf Tenson (1884–1948) (Remm 1955). The first extensive research on haematophagous Diptera in Estonia was conducted during the mid-Twentieth Century. This culminated in 1955, when H. Remm (1929–1986) completed a dissertation featuring an annotated checklist of the mosquitoes of Estonia. The manuscript was based on 12,204 specimens collected from 300 study sites, as well as available museum collections, encompassing 30 mosquito species (Remm 1955). This work was published two years later in the journal Entomologicheskoe Obozrenie (Remm 1957). Afterwards, new entries relating to mosquito species present in Estonia have been few and far between. Burtin (2014) defended a master's theses updating previous species records with currently valid synonyms and presented a study based on 691 new mosquito specimens. The manuscript included a list of 33 mosquito species likely to be present in the country, and two species suspected to occur in the country. Some of this information was later published as part of a larger study concerning urban mosquitoes, along with 1,199 additional observations (Kirik *et al.* 2021). Many of the mosquito species suspected to be present in Estonia were still missing reliable up to date records. Furthermore, information regarding the distribution and abundance of these mosquitoes had not been substantially updated after the contributions of Remm (1955). To remedy this, an updated checklist, supplemented with new evidence, was needed to better understand the mosquito fauna of the country. This would allow future researchers to track changes in species composition as well as better assess the risk of diseases caused by vector-borne pathogens in the region. Consequently, the aim of this study was to provide an updated checklist of the mosquitoes present in Estonia, along with voucher material, distribution maps, partial cytochrome c oxidase (MT-*CO1*) sequences and comments concerning the abundance of each species.

Material and methods

Study area. Estonia is the northernmost country of the three Baltic nations: it is located on the eastern shore of the Baltic Sea and shares a land border with Latvia and Russia. Estonia is situated on the East-European Plain and is therefore relatively flat, with a mean altitude of about 50 m above sea level (Raukas 1995). The country has a population density of 30.6 inhabitants per km^2, which is relatively low compared to other European nations (Eurostat 2021; Statistics Estonia 2020). Furthermore, 51.4% (relative error (RE) ±1.1%) of Estonia is consists of forests, 27.0% (RE ±1.9%) of the land is in agricultural use and bogs and inland waters make up 4.9% (RE ±5.1%) and 1.7% (RE ±8.8%) of the country, respectively (Environment Agency 2020). Estonia is considered to be part of the Boreal Region according to the European Commission (Sundseth *et al.* 2009), but belongs to the temperate continental climate zone with warm summers based on the updated Köppen-Geiger classification system (Kottek *et al.* 2006; Beck *et al*. 2018).

Mosquito collection. Adult mosquitoes were collected from 2008 to 2020 from various locations in Estonia, both from the mainland and the three largest islands: Saaremaa, Hiiumaa and Muhu (Fig. 1). Fieldwork took place from the start of May until mid-October, and included collection sites in the countryside, suburbs and urban greenspaces. In rural areas, mosquitoes were collected in farmyards, pastures, lakesides, wetlands and forest. Collection points were chosen to cover as many biomes as possible, while allowing insect traps to be emptied regularly and be supervised by volunteers. Collection sites were sampled for different periods of time due to both limited personnel and the variety of different collection methods. Most specimens were caught with the battery powered Mosquito Magnet Independence traps (Woodstream Corp., Lancaster, USA) baited with Octenol ($C_8H_{16}O$), but mosquitoes were also collected with sweep nets, EVS light traps (BioQuip Products, Rancho Dominguez, USA) baited with dry ice, Malaise traps (cf. Tomasson *et al.* 2014) and window traps (cf. Sammet *et al*. 2016). Mosquito Magnet and EVS traps were emptied every two to four days, but Malaise and window traps were emptied a few times over the summer. Information concerning the use of sweep nets can be found in a previous publication (Kirik *et al.* 2021). It is important to note that Mosquito Magnet and EVS traps use bait to attract host seeking arthropods and therefore predominantly capture female mosquitoes. As a result, the newly acquired data for the checklist primarily consists of information obtained from the collection of females.

The majority of mosquitoes were stored in tubes at -20°C as dry material, but some older samples were kept in 76% ethanol at 4°C or at room temperature. Mosquitoes were identified to species or species-group level by the first author based on morphological markers, using keys of Becker *et al.* (2020). The resulting identifications were used to make general inferences concerning the prevalence of each taxonomic group. Based on the number of individuals collected from 2008 to 2020, species were designated as abundant (>1,001 individuals), common (501–1,000 individuals), infrequent (101–500 individuals) or rare (<100 individuals) for ease of discussion. Maps showing the new and historic collection sites of each species were constructed using Adobe Photoshop CS5 Extended (Adobe, San Jose, USA) and arranged into figures. Up to three mosquitoes from every species collected were selected as voucher specimens, pinned and stored at room temperature in the Entomological Collection [IZBE] of the Estonian University of Life Sciences. The remainder of the material is also stored in the university. At least one voucher specimen of each species was subjected to DNA barcoding to further validate species identification and to help distinguish morphologically similar or isomorphic species.

DNA analysis. DNA extraction was carried out using one to three legs from each mosquito. The material was homogenized with the handheld Kontes Pellet Pestle (DWK Life Sciences GmbH, Mainz, Germany) and DNA extraction was completed using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Species identification was carried out based on the 710 bp partial sequence of the cytochrome c oxidase subunit 1 gene (*COI*), using the universal primers LCO1490 (5'- GGTCAACAAATCATAAAGATATTGG-3') and HCO2198 (5'- TAAACTTCAGGGTGACCAAAAAATCA-3') (Folmer *et al.* 1994). Additionally, 16 *Culex pipiens* mosquitoes were analyzed for the presence of the intercellular bacteria, *Wolbachia*, based on the symbionts wsp gene. This was done using primers wsp-81F (5'- TGGTCCAATAAGTGATGAAGAAAC -3') and wsp-691R (5'- AAAAATTAAACGCTACTCCA -3') (Braig *et al.* 1998). All polymerase chain reaction (PCR) mixtures consisted of 1 µl template DNA, 12.5 µl DreamTaq PCR Master Mix (Thermo Fisher Scientific, Waltham, USA), 0.5 µl of each 20 pmol/ l primer and 10.5 µl ddH_2O. For degraded material, 1.0 µl $MgCl_2$ (25 mM) (Thermo Fisher Scientific, Waltham, USA) and 0.5 µl dimethyl sulfoxide (DMSO) (ITW Reagents Division, Glenview, USA) were added as needed at the expense of ddH_2O. The PCR program for *COI* included a 15 min first denaturation stage at

94°C, followed by 60 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 44°C and 30 sec of synthesis at 72°C, capped by a 10 min final synthesis stage at 72°C. The PCR program for amplifying the *Wolbachia* wsp gene was set up according to Shaikevich *et al.* (2019b).

PCR products were checked for positive signals by electrophoresis on a 1.6% agarose gel infused with 3.8 µl of ethidium bromide, run for 1 h at 120 V and 70 mA. Six µl of each sample were mixed with 1 µl of DNA Gel Loading Dye (6X) (Thermo Fisher Scientific, Waltham, USA) before it was added to the gel. GeneRuler 100 bp DNA Ladder, ready-to-use (Thermo Fisher Scientific, Waltham, USA) was used as a reference. Successfully amplified PCR products were sequenced at the Institute of Genomics Core Facility using Sanger sequencing (University of Tartu, Tartu, Estonia). Forward and reverse nucleotide strands were combined into consensus sequences and cleaned in BioEdit version 7.2.6.1 (Hall 1999). Resulting barcodes were checked against the information stored at GenBank (National Institutes of Health, Bethesda, USA) using both the US National Library of Medicine nucleotide BLAST tool (National Institutes of Health, Bethesda, USA) and the Barcode of Life Data (BOLD) Systems workbench developed by Ratnasingham & Hebert (2007). The partial *COI* sequences of 49 voucher specimens are deposited in GenBank. The GenBank accession numbers for the species are provided for below.

Data availability. The mosquito count data generated during this research can be found online at FigShare (DOI: 10.6084/m9.figshare.16817395) or obtained from the corresponding author.

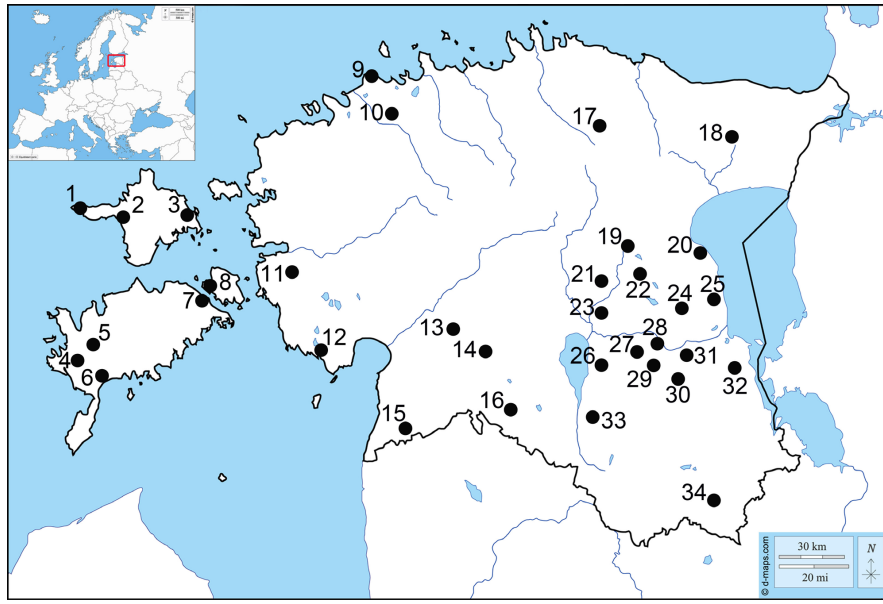

FIGURE 1. Sampling localities in Estonia: 1—Kalana, 2—Vanajõe, 3—Kerema, 4—Viidumäe, 5—Karujärve, 6—Mändjala, 7—Orissaare, 8—Igaküla, 9—Muraste, 10—Üksnurme, 11—Lihula, 12—Tõstamaa, 13—Jõesuu, 14—Punaküla, 15—Nigula NR, 16—Viivre, 17—Lasila, 18—Mäetaguse, 19—Kibuvitsa, 20—Omedu, 21—Kursi, 22—Luua, 23—Laeva, 24—Undi, 25—Kolkja, 26—Maiorg, 27—Külitse, 28—Tartu, 29—Pargi, 30—Hurda, 31—Melliste, 32—Järvselja, 33—Puka, 34—Leoski. Base maps of Europe and Estonia: © 2007–2021 https://d-maps.com (accessed on 11 August 2021).

Results

General results. The updated checklist includes 34 species based on material collected in Estonia from 2008 to 2020, information provided previously by Kirik *et al*. (2020, 2021) and historical records and studies from neighbor-

ing countries. More specifically, the newly collected material included in this study consists of 24,344 adult mosquitoes (94.2% female), which by themselves helped confirm the presence of 27 species. Most of these mosquitoes were identified based on their morphological characteristics, but some were also submitted for genetic identification based on mitochondrial *COI* sequences. All mosquitoes that were collected belong to one or other of five genera: *Aedes*, *Anopheles*, *Coquillettidia*, *Culex* and *Culiseta*. In the following checklist, information on mosquito species in Estonia is summarized and annotated with brief comments. Regrettably, the historical material underlying the first checklist compiled by Remm (1957) no longer exists; thus, seven species not encountered during the recent collections, are included based on only literature sources.

Annotated checklist

Genus *Aedes* Meigen, 1818

1. *Aedes* (*Aedes*) *cinereus* Meigen, 1818
(Fig. 2A)

Published sources: Remm (1957: 156), Burtin (2014: 33), Khalin *et al*. (2020: 61), Kirik *et al*. (2021: 11).

Voucher material: 1♀, Tartu (58° 21′ 26″ N, 26° 42′ 60″ E), 14.VI.2017, T. Kesküla leg., H. Kirik det., sweep net, IZBE0210180; 1♂, Tartu (58° 21′ 23″ N, 26° 44′ 31″ E), 24.IX.2017, T. Kesküla leg., H. Kirik det., sweep net, IZBE0210247; 1♀, Tartu (58° 23′ 24″ N, 26° 42′ 55″ E), 14.IX.2016, H. Kirik leg., H. Kirik det., sweep net, IZBE0210181, GenBank: OK465139.

Comment: 1,436 mosquitoes (5.9% of all specimens collected) were identified as *Ae. cinereus*. This species is abundant in Estonia and can be found almost everywhere from June to September. Also, *Ae. cinereus* can be numerous at times, especially towards the end of the summer, based on the data of the present study. It should be noted that adult females are difficult to differentiate from the closely related *Ae. geminus* based on morphology alone; thus, specimens of *Ae. geminus* could also be among the specimens of *Ae. cinereus* collected during the study.

2. *Aedes* (*Aedimorphus*) *vexans* (Meigen, 1830)
(Fig. 2B)

Published sources: Remm (1957: 156), Burtin (2014: 34), Lilja *et al*. (2018: 283), Kirik *et al*. (2021: 11).

Voucher material: 1♀, Tartu (58° 21′ 23″ N, 26° 44′ 31″ E), 13.VIII.2017, T. Kesküla leg., H. Kirik det., sweep net, IZBE0210182, GenBank: OK465140; 1♀, Tartu (58° 22′ 17″ N, 26° 41′ 58″ E), 21.VIII.2017, H. Kirik leg., H. Kirik det., sweep net, IZBE0210183, GenBank: OK465141; 1♂, Tartu (58° 21′ 16″ N, 26° 40′ 53″ E), 06.VI.2015, T. Kesküla leg., O. Kurina det., sweep net, IZBE0210184.

Comment: 366 mosquitoes (1.5% of all specimens collected) were identified as *Ae. vexans*. This species was infrequently collected, but specimens could be found throughout the warm season, with a peak of activity in August. However, *Ae. vexans* has been found to emerge in large numbers after floods (Schäfer & Lundström 2009), so their low numbers in this study could be due to sampling bias.

3. *Aedes* (*Ochlerotatus*) *annulipes* (Meigen, 1830)
(Fig. 2C)

Published sources: Remm (1957: 154), Burtin (2014: 43), Kirik *et al*. (2021: 11, as part of the *Aedes* (*Ochlerotatus*) *annulipes* group).

Voucher material: 1♀, Mändjala (58° 12′ 56″ N, 22° 19′ 56″ E), 16.VI.2015, L. Tummeleht leg., H. Kirik det., Mosquito Magnet trap, IZBE0210241, GenBank: OK465167; 1♂, Omedu (58° 45′ 09″ N, 27° 02′ 23″ E), 06.VI.2015, O. Kurina leg., O. Kurina det., sweep net, IZBE021218.

Comment: *Aedes annulipes* belongs to the *Ae. annulipes* group, along with *Ae. cantans* (Meigen, 1818), *Ae.*

cyprius Ludlow, 1920, *Ae. euedes* Howard, Dyar & Knab, 1913, *Ae. excrucians* (Walker, 1956), *Ae. flavescens* (Müller, 1764), *Ae. riparius* Dyar & Knab, 1907, etc. (Becker *et al*. 2020). *Aedes annulipes* can be morphologically distinguished from others species of the group, but this can be difficult when it comes to adult females. This is because of the variability in their morphological traits as well as inconclusive DNA evidence, making species identification time and resource extensive. For the purposes of this study, mosquitoes with morphology similar to *Ae. annulipes* were designated as specimens of the *Ae. annulipes* group. In total, 2,091 individuals (8.6% of all specimens collected) were identified as simply belonging to the group. These mosquitoes were active from May to October, but were most numerous in June. Two specimens, which corresponded well to both the morphological description of *Ae. annulipes* and the partial *COI* sequences found in online databases, were chosen as the local voucher specimens.

4. *Aedes* (*Ochlerotatus*) *cantans* (Meigen, 1818)
(Fig. 2C)

Published sources: Remm (1957: 152, as *Aëdes maculatus* Meigen, 1804), Burtin (2014: 43), Kirik *et al.* (2021: 11, under the *Aedes* (*Ochlerotatus*) *annulipes* group).

Voucher material: 1♀, Tartu (58° 22′ 17″ N, 26° 41′ 58″ E), 06.VI.2017, H. Kirik leg., H. Kirik det., sweep net, IZBE0210215, GenBank: OK465165; 1♂, Tartu (58° 23′ 54″ N, 26° 44′ 40″ E), 17.V.2015, O. Kurina leg., O. Kurina det., sweep net, IZBE0210216; 1♀, Tartu (58° 21′ 1″ N, 26° 41′ 30″ E), 24.VI.2017, T. Kesküla leg., H. Kirik det., sweep net, IZBE0210217, GenBank: OK465166.

Comment: *Aedes cantans* belongs to the *Ae. annulipes* group. The species of this group can be distinguished based on morphological characteristics, but due to the variability of some traits, as well as inconclusive results of DNA barcoding, mosquitoes similar to *Ae. cantans* were designated as specimens of the *Ae. annulipes* group. In all, 2,091 mosquitoes (8.6% of all specimens collected) were as belonging to the *Ae. annulipes* group. These individuals were found throughout the warm months, but were most numerous in June. Voucher material was chosen from among specimens that best corresponded to the morphological traits of *Ae. cantans* and matched well with reliable DNA sequences in online databases.

5. *Aedes* (*Ochlerotatus*) *caspius* (Pallas, 1771)
(Fig. 2D)

Published source: Remm (1957: 152).

Voucher material: 1♀, Vanajõe (58° 53′ 16″ N, 22° 26′ 37″ E), 20–21.VIII.2015, L. Tummeleht leg., H. Kirik det., Mosquito Magnet trap, IZBE0210185, GenBank: OK465142; 1♂, Kerema (58° 53′ 26″ N, 26° 56′ 52″ E), 05–19.VII.2009, R. Miller leg., H. Kirik det., Malaise trap, IZBE0210187; 1♀, Vanajõe (58° 53′ 16″ N, 22° 26′ 37″ E), 21–22.VIII.2015, L. Tummeleht leg., H. Kirik det., Mosquito Magnet trap, IZBE0210186.

Comment: 206 mosquitoes (0.9% of all specimens collected) were identified as *Ae. caspius*. *Aedes caspius* is a halophilic species mainly found in Estonia near the brackish water of the Baltic Sea. These mosquitoes are common and at times numerous near the coastline, but are rarely found further inland. Thus, their relatively low numbers collected in this study is due to collection bias. *Aedes caspius* appears to be active throughout summer.

6. *Aedes* (*Ochlerotatus*) *cataphylla* Dyar, 1916
(Fig. 2E)

Published sources: Remm (1957: 154), Khalin *et al*. (2020: 66), Kirik *et al*. (2021: 11).

Voucher material: 1♀, Tartu (58° 23′ 44″ N, 26° 43′ 44″ E), 06.VI.2017, H. Kirik leg., H. Kirik det., sweep net, IZBE0210188, GenBank: OK465143; 1♀, Tartu (58° 20′ 52″ N, 26° 41′ 37″ E), 14.VI.2017, T. Kesküla leg., H. Kirik det., sweep net, IZBE0210189, GenBank: OK465144; 1♂, Tartu (58° 23′ 40″ N, 26° 44′ 05″ E), 14.VI.2017, H. Kirik leg., H. Kirik det., sweep net, IZBE0210244.

Comment: 3,951 mosquitoes (16.2% of all specimens collected) were identified as *Ae. cataphylla*, making it

the third most common mosquito species in Estonia. This species is abundant almost everywhere in the country and can be numerous at times. As is typical for a spring-time species, *Ae. cataphylla* are most active in May, but some specimens can be found until September.

7. *Aedes* (*Ochlerotatus*) *communis* (de Geer, 1776)
(Fig. 2F)

Published sources: Remm (1957: 155), Burtin (2014: 44), Kirik *et al*. (2021: 11).

Voucher material: 1♀, Tõrve (58° 37′ 40″ N, 26° 23′ 48″ E), 27.VI–01.VII.2018, H. Kirik leg., H. Kirik det., Mosquito Magnet trap, IZBE0210190, GenBank: OK465145; 1♀, Tartu (58° 23′ 40″ N, 26° 44′ 05″ E), 06.VI.2017, H. Kirik leg., H. Kirik det., sweep net, IZBE0210191, GenBank: OK465146; 1♂, Tartu (58° 23′ 24″ N, 26° 44′ 40″ E), 17.V.2015, O. Kurina leg., O. Kurina det., sweep net, IZBE0210192.

Comment: 7,316 mosquitoes (30.1% of all specimens collected) were identified as *Ae. communis*, making it the dominant species in Estonia. *Aedes communis* can be found everywhere in the country. It is especially numerous during May and June, but individuals can be found until October. Importantly, there appears to be two distinct mitochondrial lineages in the area, which can make DNA barcoding difficult, as some *COI* sequences appear to be very similar to the North American *Ae. tahoensis* (Dyar, 1916) (Kirik *et al.* 2020).

8. *Aedes* (*Ochlerotatus*) *cyprius* Ludlow, 1920
(Fig. 2G)

Published source: Remm (1957: 153).

Voucher material: 1♀, Lasila (59° 16′ 47″ N, 26° 13′ 24″ E), 31.V.2016, M. Kruus leg., H. Kirik det., Malaise trap, IZBE0210193, GenBank: OK465147; 1♀, Lasila (59° 16′ 47″ N, 26° 13′ 24″ E), 31.V.2016, M. Kruus leg., H. Kirik det., Malaise trap, IZBE0210194, GenBank: OK465148.

Comment: Four mosquitoes were identified as *Ae. cyprius*. It was very rare among the mosquitoes collected during this study, but it can be found at the beginning of the warm season in May and June. The only individuals collected in the country from 2008 to 2020 were caught in northern Estonia. The low numbers captured could also be due to collection bias.

9. *Aedes (Ochlerotatus) diantaeus* Howard, Dyar & Knab, 1913
(Fig. 2H)

Published sources: Remm (1957: 154), Burtin (2014: 45), Kirik *et al*. (2021: 11).

Voucher material: 1♂, Tartu (58° 23′ 44″ N, 26° 43′ 44″ E), 07.VI.2013, V. Burtin leg., V. Burtin det., sweep net, IZBE0210010.

Comment: In this study, only one mosquito was identified as *Ae. diantaeus*. This single specimen was collected in southeastern Estonia at the beginning of June. More research is needed to better understand the abundance of this species in the country.

10. *Aedes (Ochlerotatus) dorsalis* (Meigen, 1830)
(Fig. 3A)

Published source: Remm (1957: 152).

Voucher material: None.

Comment: One male and four females of *Ae. dorsalis* have been reported from Estonia (Remm 1957). However, the specimens have not been preserved and cannot be verified. No specimens were found during the present study. *Aedes dorsalis* has been reported from Lithuania (Pakalniškis *et al.* 2000), Latvia (Spungis 2000), provinces

adjacent to Estonia in northwestern Russia (Khalin & Aibulatov 2020), Finland (Culverwell 2018; Culverwell *et al*. 2021) and Sweden (Lundström *et al.* 2013). Therefore, this species is likely to be present in Estonia as well. The absence of specimens in collections made during this study could be due to insufficient trapping, as well as collection bias, as *Ae. dorsalis* is halophilic and has been known to be associated with floods (Becker *et al.* 2020).

11. *Aedes* (*Ochlerotatus*) *excrucians* (Walker, 1856)
(Fig. 3B)

Published sources: Remm (1957: 154), Burtin (2014: 46), Kirik *et al*. (2021: 11).

Voucher material: 1♀, Tipu (58° 21′ 47″ N, 25° 3′ 29″ E), 31.V–03.VI.2018, H. Kirik leg., H. Kirik det., Malaise trap, IZBE0210195, GenBank: OK465149; 1♀, Tartu (58° 21′ 13″ N, 26° 40′ 45″ E), 24.VI.2017, T. Kesküla leg., H. Kirik det., sweep net, IZBE0210196, GenBank: OK465150; 1♂, Tartu (58° 23′ 23″ N, 26° 41′ 58″ E), 30.V.2015, O. Kurina leg., O. Kurina det., sweep net, IZBE0210197.

Comment: 193 mosquitoes (0.8% of all collected specimens collected) were identified as *Ae. excrucians*. The species appears to be uncommon in the country based on this study, but individuals can be found from May to October, although the peak of their activity seems to be in June.

12. *Aedes* (*Ochlerotatus*) *flavescens* (Müller, 1764)
(Fig. 3C)

Published sources: Remm (1957: 153), Burtin (2014: 46), Kirik *et al*. (2021: 11).

Voucher material: 1♀, Undi (58° 29′ 44″ N, 26° 54′ 00″ E), 15–16.VIII.08.2016, L. Tummeleht leg., H. Kirik det., Mosquito Magnet trap, IZBE0210198, GenBank: OK465151; 1♀, Marja (58° 23′ 10″ N, 26° 42′ 39″ E), 14–15.VI.2016, O. Kurina leg., H. Kirik det., Mosquito Magnet trap, IZBE0210199, GenBank: OK465152; 1♀, Marja (58° 23′ 10″ N, 26° 42′ 39″ E), 19–21.VI.2015, O. Kurina leg., H. Kirik det., Mosquito Magnet trap, IZBE0210200, GenBank: OK465153.

Comment: 77 mosquitoes (0.3% of all specimens collected) were identified as *Ae. flavescens*. This species seems to be rare in Estonia, but specimens can be found throughout the warm season, from May to October. It is slightly more numerous in May.

13. *Aedes* (*Ochlerotatus*) *hexodontus* Dyar, 1916
(Fig. 3D)

Published sources: None.

Voucher material: 1♀, Tõrve (58° 35′ 56″ N, 26° 22′ 20″ E), 27.VI–01.VII.2018, H. Kirik leg., H. Kirik det., Mosquito Magnet trap, IZBE0210201, GenBank: OK465154; 1♀, Undi (58° 29′ 44″ N, 26° 54′ 00″ E), 15–16.V.2016, L. Tummeleht leg., H. Kirik det., Mosquito Magnet trap, IZBE0210202, GenBank: OK465155; 1♀, Undi (58° 29′ 44″ N, 26° 54′ 00″ E), 22–23.V.2015, L. Tummeleht leg., H. Kirik det., Mosquito Magnet trap, IZBE0210203, GenBank: OK465156.

Comment: 35 mosquitoes (0.1% of all specimens collected) were identified as *Ae. hexodontus*. This species appears to be relatively rare in Estonia and from 2008 to 2020 it was only collected in May and June.

14. *Aedes* (*Ochlerotatus*) *intrudens* Dyar, 1919
(Fig. 3E)

Published sources: Remm (1957: 156), Burtin (2014: 47), Kirik *et al*. (2021: 11).

Voucher material: 1♀, Tartu (58° 21′ 26″ N, 26° 42′ 60″ E), 04.VI.2017, T. Kesküla leg., H. Kirik det., sweep net, IZBE0210204, GenBank: OK465157; 1♀, Viivre (58° 04′ 52″ N, 25° 31′ 26″ E), 18–19.VI.2016, H. Kirik leg.,

H. Kirik det., Mosquito Magnet trap, IZBE0210205, GenBank: OK465158; 1♂, Tartu (58° 23′ 24″ N, 26° 44′ 40″ E), 17.V.2015, O. Kurina leg., O. Kurina det., sweep net, IZBE0210206.

Comment: 189 mosquitoes (0.8% of all specimens collected) were identified as *Ae. intrudens*. This species was uncommon in Estonia during the fieldwork of this study. *Aedes intrudens* appears to be active from May to July, but it is more numerous in May.

15. *Aedes* (*Ochlerotatus*) *leucomelas* (Meigen, 1804)
(Fig. 3F)

Published sources: Remm (1957: 154), Kirik *et al*. (2021: 11).

Voucher material: 1♀, Tõrve (58° 37′ 32″ N, 26° 23′ 24″ E), 26–29.V.2018, H. Kirik leg., H. Kirik det., Mosquito Magnet trap, IZBE0210207, GenBank: OK465159; 1♀, Tartu (58° 21′ 26″ N, 26° 42′ 60″ E), 14.06.2017, T. Kesküla leg., H. Kirik det., sweep net, IZBE0210208, GenBank: OK465160; 1♀, Tartu (58° 21′ 36″ N, 26° 43′ 56″ E), 19.VI.2017, T. Kesküla leg., H. Kirik det., sweep net, IZBE0210209.

Comment: 211 mosquitoes (0.9% of all specimens collected) were identified as *Ae. leucomelas*. This species seems to be uncommon in Estonia, much less common than the closely related *Ae. cataphylla*. Mosquitoes identified as *Ae. leucomelas* were most numerous in May, but some individuals were found until October during this study.

16. *Aedes* (*Ochlerotatus*) *nigrinus* (Eckstein, 1918)
(Fig. 3G)

Published sources: Remm (1957: 155), Burtin (2014: 48).

Voucher material: None.

Comment: Three males and 23 females of *Ae. nigrinus* were previously reported from Estonia (Remm 1957), but the specimens have not been preserved. No specimens were collected during this study. However, *Ae. nigrinus* has also been recorded in Lithuania (Pakalniškis *et al.* 2000), provinces in northwestern Russia adjacent to Estonia (Khalin & Aibulatov 2020), Finland (Harbach *et al.* 2017; Culverwell *et al*., 2021) and Sweden (Lundström *et al.* 2013). Therefore, this species is likely to be present in Estonia as well.

17. *Aedes* (*Ochlerotatus*) *punctor* (Kirby, 1837)
(Fig. 3H)

Published sources: Remm (1597: 155), Burtin (2014: 48), Kirik *et al*. (2021: 11).

Voucher material: 1♀, Tartu (58° 22′ 52″ N, 26° 42′ 49″ E), 06.VI.2017. H. Kirik leg., H. Kirik det., sweep net, IZBE0210210, GenBank: OK465161; 1♀, Tartu (58° 22′ 52″ N, 26° 42′ 49″ E), 19.VI.2017, H. Kirik leg., H. Kirik det., sweep net, IZBE0210211, GenBank: OK465162; 1♂, Tartu (58° 23′ 54″ N, 26° 44″ 40'' E), 17.V.2015, O. Kurina leg., O. Kurina det., sweep net, IZBE0210212.

Comment: 4,594 mosquitoes (18.9% of all specimens collected) were grouped as *Ae. punctor*/*punctodes* Dyar, 1922, although males were identified as *Ae. punctor*. *Aedes punctor* is known to be more common than *Ae. punctodes* (Culverwell *et al*., 2021), but further DNA analysis or larval collections are required to make definitive conclusions about the presence of *Ae. punctodes* in Estonia. The two females of *Ae. punctor*/*punctodes* chosen as voucher specimens were identified as *Ae. punctor* by DNA barcoding. These mosquitoes were especially numerous in May during this study, but some individuals were found until October.

18. *Aedes (Ochlerotatus) riparius* Dyar & Knab, 1907
(Fig. 4B)

Published source: Remm (1957: 153).

Voucher material: None.

Comment: *Aedes riparius* belongs to the *Ae. annulipes* group and can be difficult to identify. A specimen corresponding to morphologically to *Ae. riparius* was found during this study, but DNA barcoding identified it as *Ae. annulipes*/*cantans*. One male, four females and one larva of *Ae. riparius* have previously been reported from Estonia (Remm 1957), but these individuals have not been preserved and their identification cannot be verified. *Aedes riparius* has been found in Lithuania (Pakalniškis *et al.* 2000), Latvia (Spungis 2000), provinces in Northwestern Russia adjacent to Estonia (Khalin & Aibulatov 2020), Finland (Culverwell 2018; Culverwell *et al*. 2021) and Sweden (Lundström *et al.* 2013); consequently, *Ae. riparius* is likely present in Estonia as well.

19. *Aedes* (*Ochlerotatus*) *sticticus* (Meigen, 1838)
(Fig. 4A)

Published source: Kirik *et al*. (2021: 11).

Voucher material: 1♀, Külitse (58° 20′ 5″ N, 26° 35′ 57″ E), 01–03.VIII.2020, V. Oborina leg., H. Kirik det., Mosquito Magnet trap, IZBE0210213, GenBank: OK465163; 1♂, Tartu (58° 20′ 52″ N, 26° 41′ 37″ E), 17.07.2017, T. Kesküla leg., H. Kirik det., sweep net, IZBE0210249; 1♀, Tartu (58° 23′ 40″ N, 26° 44′ 05″ E), 05.VII.2017, H. Kirik leg., H. Kirik det., sweep net, IZBE0210214, GenBank: OK465164.

Comment: 231 mosquitoes (1.0% of all specimens collected) were identified as *Ae. sticticus*. This species appears to be uncommon in the country based on the current collections. However, the abundance of *Ae. sticticus* has been found to depend on floods, and they can be numerous at certain times (Schäfer & Lundström 2009). Their relatively low numbers in this study could be due to collection bias. Interestingly, *Ae. sticticus* was encountered more often in August, which is unusual compared to most *Aedes* species in the area. Overall, some *Ae. sticticus* can be found in Estonia throughout the summer, from June to September.

Genus *Anopheles* Meigen, 1818

20. *Anopheles* (*Anopheles*) *algeriensis* Theobald, 1903
(Fig. 4C)

Published source: Remm (1957: 150).

Voucher material: None.

Comment: Nine *An. algeriensis* females have been previously reported from Estonia, but these specimens have not been preserved and cannot be verified. No new specimens were found during this study. Also, Sweden is the closest country to Estonia where *An. algeriensis* has been collected (Lundström *et al.* 2013). In fact, this species appears to be most common in Mediterranean and Balkan countries (Scholte *et al.* 2011).

21. *Anopheles* (*Anopheles*) *claviger* (Meigen, 1804)
(Fig. 4D)

Published sources: Remm (1957: 150, as *Anopheles bifurcatus* Linnaeus, 1758), Burtin (2014: 35), Kirik *et al*. (2021: 11).

Voucher material: 1♀, Ülenurme (58° 19′ 3″ N, 26° 43′ 23″ E), 14–17.VIII.2020, H. Kirik leg., H. Kirik det., Mosquito Magnet trap, IZBE0210219, GenBank: OK465168; 1♀, Undi (58° 29′ 44″ N, 26° 54′ 00″ E), 15–16.VIII.2016, L. Tummeleht leg., H. Kirik det., Mosquito Magnet trap, IZBE0210220, GenBank: OK465169; 1♂, Tartu (58° 23′ 24″ N, 26° 42′ 55″ E), 03.IX.2013, V. Burtin leg., V. Burtin det., sweep net, IZBE0210007.

Comment: 1,038 mosquitoes (4.3% of all specimens collected) were identified as *An. claviger*. This species is abundant in Estonia, especially during August, but some individuals can be found from May to October. *Anopheles claviger* was the most common anopheline mosquito in this study.

22. *Anopheles* (*Anopheles*) *maculipennis* Meigen, 1818 *s.s.*
(Fig. 4E)

Published sources: Remm (1957: 151), Burtin (2014: 36, as *An. maculipennis s.l.*), Kirik *et al*. (2021: 11, part of the *An. maculipennis* complex).

Voucher material: 1♂, Ülenurme (58° 23′ 40″ N, 26° 44′ 05″ E), 19.VIII.2016, H. Kirik leg., H. Kirik det., sweep net, IZBE0210243, GenBank: OK465173.

Comment: *Anopheles maculipennis* is the nominotypical species of the *An. maculipennis* complex. Three mosquitoes were identified as *An. maculipennis s.s* among of 20 specimens of the *An. maculipennis* complex that were subjected to DNA barcoding. These results indicate that *An. maculipennis* is likely to be quite uncommon in the country, as only 215 mosquitoes (0.9% of all collected specimens collected) were identified as belonging to the *An. maculipennis* complex, and presumably *An. maculipennis* makes up a small portion of these individuals. The true abundance of the *An. maculipennis* complex is likely to have been underestimated in this study due to collection bias.

23. *Anopheles* (*Anopheles*) *messeae* Falleroni, 1926
(Fig. 4E)

Published source: Kirik *et al*. (2020: 5).

Voucher material: 1♀, Punaküla (58° 20′ 9″ N, 25° 20′ 4″ E), 31.V.–03.VI.2018, H. Kirik leg., H. Kirik det., Mosquito Magnet trap, IZBE0210221, GenBank: OK465170; 1♀, Tartu (58° 21′ 26″ N, 26° 42′ 60″ E), 04.IX.2017, T. Kesküla leg., H. Kirik det., sweep net, IZBE0210222, GenBank: OK465171; 1♀, Tartu (58° 21′ 13″ N, 26° 40′ 45” E), 17.IX.2017, T. Kesküla leg., H. Kirik det., sweep net, IZBE0210223, GenBank: OK465172.

Comment: *Anopheles messeae* belongs to the *An. maculipennis* complex. Based on *COI* sequences, 17 mosquitoes among 20 specimens of the complex subjected to genetic analyses were identified as *An. messeae* or *An. daciae* Linton, Nicolescu & Harbach, 2004 (in Nicolescu *et al*. 2004). This indicates that *An. messeae*/*daciae* most likely make up the majority of the 215 (0.9% of all specimens collected) specimens of the complex collected in this study. *Anopheles messeae* and *An. daciae* are difficult to distinguish based on *COI* sequences at this time, but the ribosomal internal transcribed spacer 2 (ITS2) region of one Estonian *An. messeae* specimen was sequenced in a previous study (Kirik *et al.* 2020). All in all, individuals belonging to the *An. maculipennis* complex could be found from May to October, but were more numerous in August. The abundance of mosquitoes of the complex may be underestimated in this study due to collection bias.

24. *Anopheles* (*Anopheles*) *plumbeus* Stephens, 1828
(Fig. 4F)

Published source: Remm (1957: 150).

Voucher material: None.

Comment: There are historical records of four *An. plumbeus* females collected from Estonia (Remm 1957). No specimens were identified during this study, but this may be because of collection bias. *Anopheles plumbeus* has also been reported from Lithuania (Pakalniškis *et al.* 2000) and Sweden (Lundström *et al.* 2013), but not from other countries neighboring Estonia.

Genus *Coquillettidia* Dyar, 1904

25. *Coquillettidia* (*Coquillettidia*) *richiardii* (Ficalbi, 1889)
(Fig. 4G)

Published sources: Remm (1957: 152, as *Mansonia richiardii*), Burtin (2014: 38), Kirik *et al*. (2021: 11).

Voucher material: 1♀, Undi (58° 29′ 44″ N, 26° 54′ 00″ E), 16–17.VIII.2015, L. Tummeleht leg., H. Kirik det., Mosquito Magnet trap, IZBE0210224, GenBank: OK465174; 1♀, Undi (58° 29′ 44″ N, 26° 54′ 00″ E), 16–17.VIII.2015, L. Tummeleht leg., H. Kirik det., Mosquito Magnet trap, IZBE0210225, GenBank: OK465175; 1♂, Tartu (58° 23′ 20″ N, 26° 42′ 52″ E), 31.VII.2017, H. Kirik leg., H. Kirik det., sweep net, IZBE0210251.

Comment: 787 mosquitoes (3.2% of all specimens collected) were identified as *Cq. richiardii*. This species is common in the country and specimens have been found from June to October, with a peak of activity in July.

Genus *Culex* Linnaeus, 1758

26. *Culex* (*Culex*) *pipiens* Linnaeus, 1758

(Fig. 4H)

Published sources: Remm (1957: 157), Burtin (2014: 39), Kirik *et al*. (2021: 11, as *Cx.* (*Cux.*) *pipiens*/*torrentium*).

Voucher material: 1♀, Tartu (58° 21′ 23″ N, 26° 44′ 31″ E), 24.IX.2017, T. Kesküla leg., H. Kirik det., sweep net, IZBE0210226, GenBank: OK465176; 1♂, Tartu (58° 23′ 05″ N, 26° 42′ 19″ E), 27.IX.2016, H. Kirik leg., H. Kirik det., sweep net, IZBE0210248; 1♀, Tartu (58° 21′ 26″ N, 26° 42′ 60″ E), 28.VIII.2017, T. Kesküla leg., H. Kirik det., sweep net, IZBE0210227, GenBank: OK465177.

Comment: Adults of *Cx. pipiens* are difficult to distinguish from *Cx. torrentium* by morphological characteristics alone. However, when 12 *Cx. pipiens*/*torrentium* females were subjected to DNA barcoding, five (41.7%) were identified as *Cx. pipiens*. In 2013, 64 (48.5%) male mosquitoes were identified as *Cx. pipiens* compared to 68 (51.5%) determined to be *Cx. torrentium*. In 2017. however, 84 (60.9%) males were identified as *Cx. pipiens* and only 54 (39.1%) were identified as *Cx. torrentium*. Based on this information, *Cx. pipiens* and *Cx. torrentium* could be present in relatively similar numbers in Estonia. It is possible that the true relative abundance of *Cx. pipiens* is underestimated in this study due to collection bias. Mosquitoes identified as *Cx. pipiens*/*torrentium* were most numerous in September. Also, *Cx. pipiens* specimens in Estonia were found to be infected with the intercellular symbiont *Wolbachia pipientis*, which agrees with the published literature (Bergman & Hesson 2021; Inácio da Silva *et al.* 2021). No attempts were made to identify the "molestus" biotype of *Cx. pipiens* among the specimens collected during the study.

27. *Culex* (*Culex*) *torrentium* Martini, 1925

(Fig. 4H)

Published sources: Remm (1057: 157, as *Culex exilis* Dyar, 1924), Burtin (2014: 40), Kirik *et al*. (2021: 11, as *Cx.* (*Cux.*) *pipiens*/*torrentium*).

Voucher material: 1♀, Tartu (58° 22′ 17″ N, 26° 41′ 58″ E), 27.IX.2016, H. Kirik leg., H. Kirik det., sweep net, IZBE0210228, GenBank: OK465178; 1♀, Tartu (58° 23′ 40″ N, 26° 44′ 05″ E), 08.IX.2016, H. Kirik leg., H. Kirik det., sweep net, IZBE0210229, GenBank: OK465179; 1♂, Tartu (58° 23′ 44″ N, 26° 43′ 44″ E), 27.IX.2016, H. Kirik leg., H. Kirik det., sweep net, IZBE0210245.

Comment: The adult females of *Cx. torrentium* are difficult to distinguish from *Cx. pipiens* based on only morphological characteristics. Of 12 females subjected to DNA barcoding, seven (58.3%) were identified as *Cx. torrentium*. Adult males of the two species can be distinguished based on structures of their genitalia. In 2013, 68 (51.5%) among 132 males were identified as *Cx. torrentium*. In 2017, only 54 (39.1%) males were determined to be *Cx. torrentium* compared to 84 (60.9%) of individuals identified as *Cx. pipiens*. All things considered, it is reasonable to assume that *Cx. torrentium* makes up about half of the 1,236 (5.1% of all collected mosquitoes collected) identified as *Cx. pipiens/torrentium* during in this study. However, the true relative abundance of these mosquitoes may have been underestimated in this study due to collection bias. *Culex torrentium* and *Cx. pipiens* are most active at the end of summer, when they become dominant. Seven *Cx. torrentium* caught in Estonia were analyzed for *Wolbachia pipientis* using the wsp gene for detection. The results were negative, which is in line with the findings of Bergman & Hesson (2021).

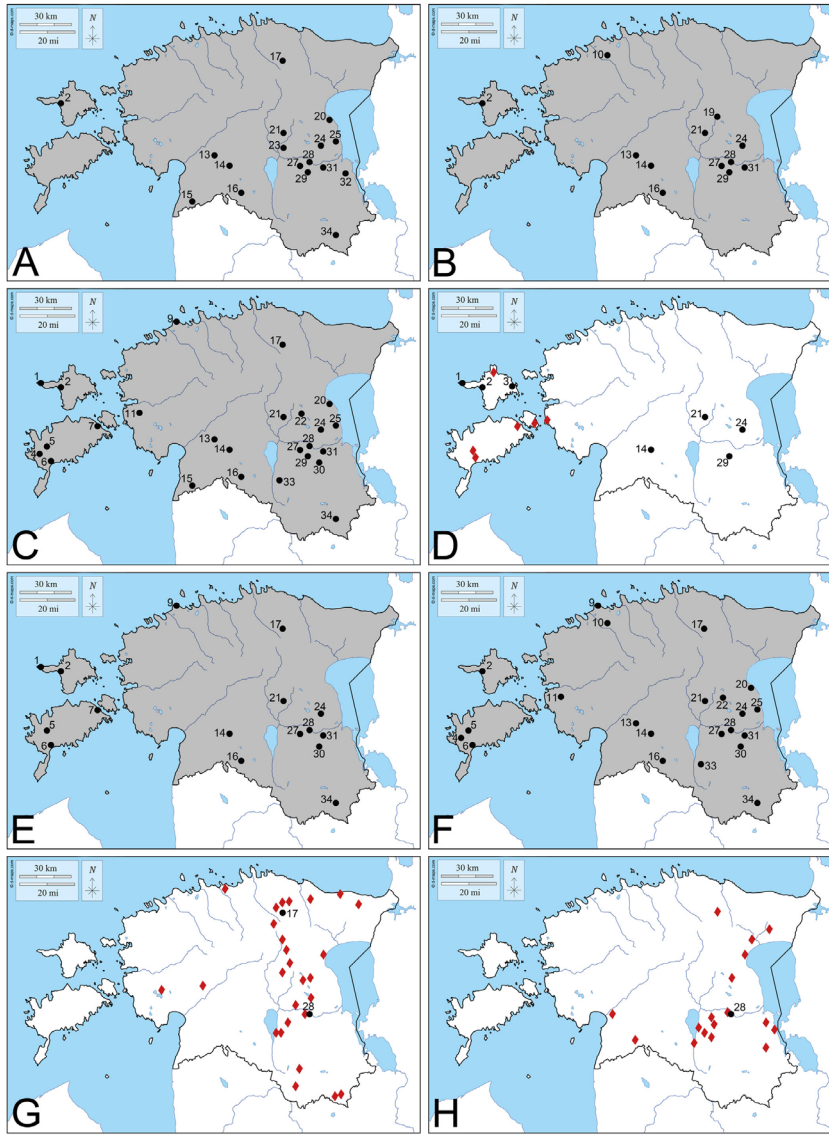

FIGURE 2. Maps showing the historic and current collection points of individual mosquito species in Estonia. **A**, *Aedes* (*Aedes*) *cinereus*; **B**, *Aedes* (*Aedimorphus*) *vexans*; **C**, *Aedes annulipes* group; **D**, *Aedes* (*Ochlerotatus*) *caspius*; **E**, *Aedes* (*Ochlerotatus*) *cataphylla*; **F**, *Aedes* (*Ochlerotatus*) *communis*; **G**, *Aedes* (*Ochlerotatus*) *cyprius*; **H**, *Aedes* (*Ochlerotatus*) *diantaeus*. Numbers indicate original data and correspond to on the numbers in Fig. 1. Red diamonds indicate localities collected by Remm (1955). In cases where Remm indicated the species was widely distributed in Estonia, the area is shaded in gray.

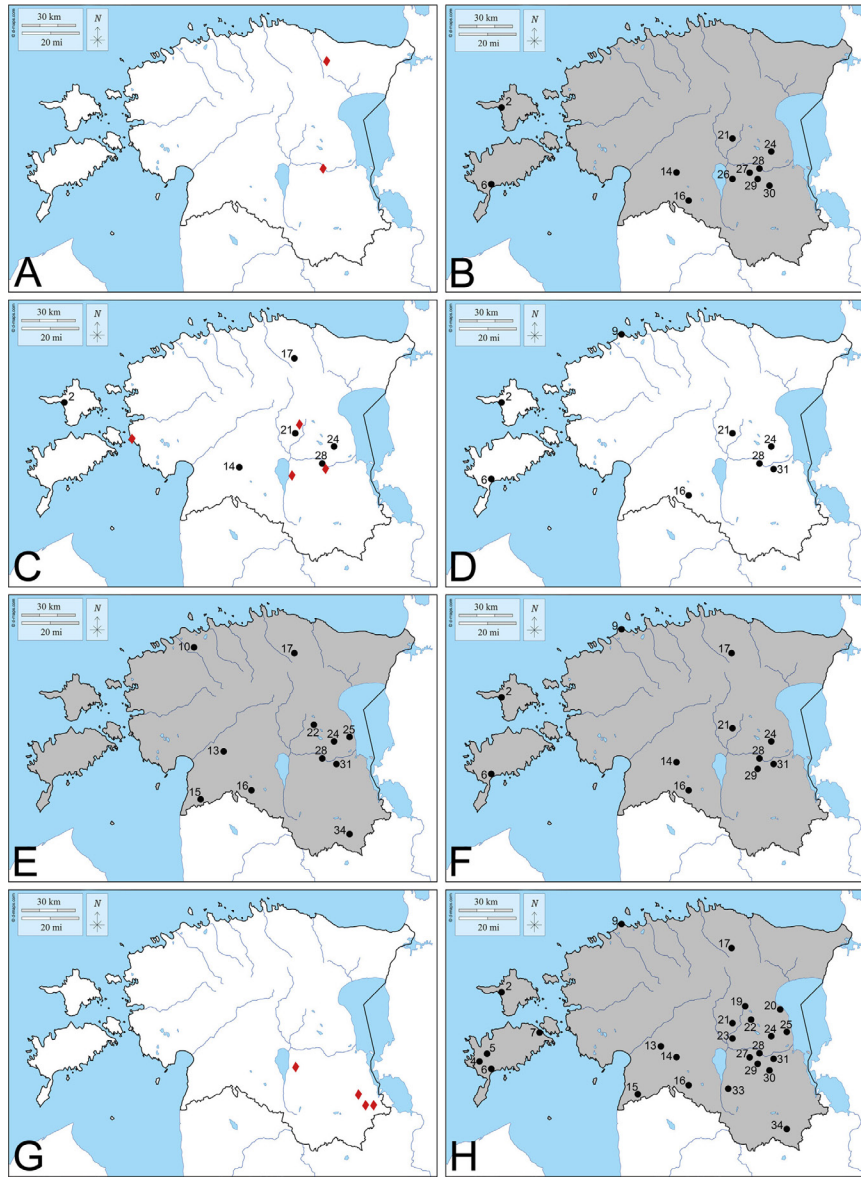

FIGURE 3. Maps showing the historic and current collection points of individual mosquito species in Estonia. **A**, *Aedes* (*Ochlerotatus*) *dorsalis*; **B**, *Aedes* (*Ochlerotatus*) *excrucians*; **C**, *Aedes* (*Ochlerotatus*) *flavescens*; **D**, *Aedes* (*Ochlerotatus*) *hexodontus*; **E**, *Aedes* (*Ochlerotatus*) *intrudens*; **F**, *Aedes* (*Ochlerotatus*) *leucomelas*; **G**, *Aedes* (*Ochlerotatus*) *nigrinus*; **H**, *Aedes* (*Ochlerotatus*) *punctor*. For details, see Fig. 2.

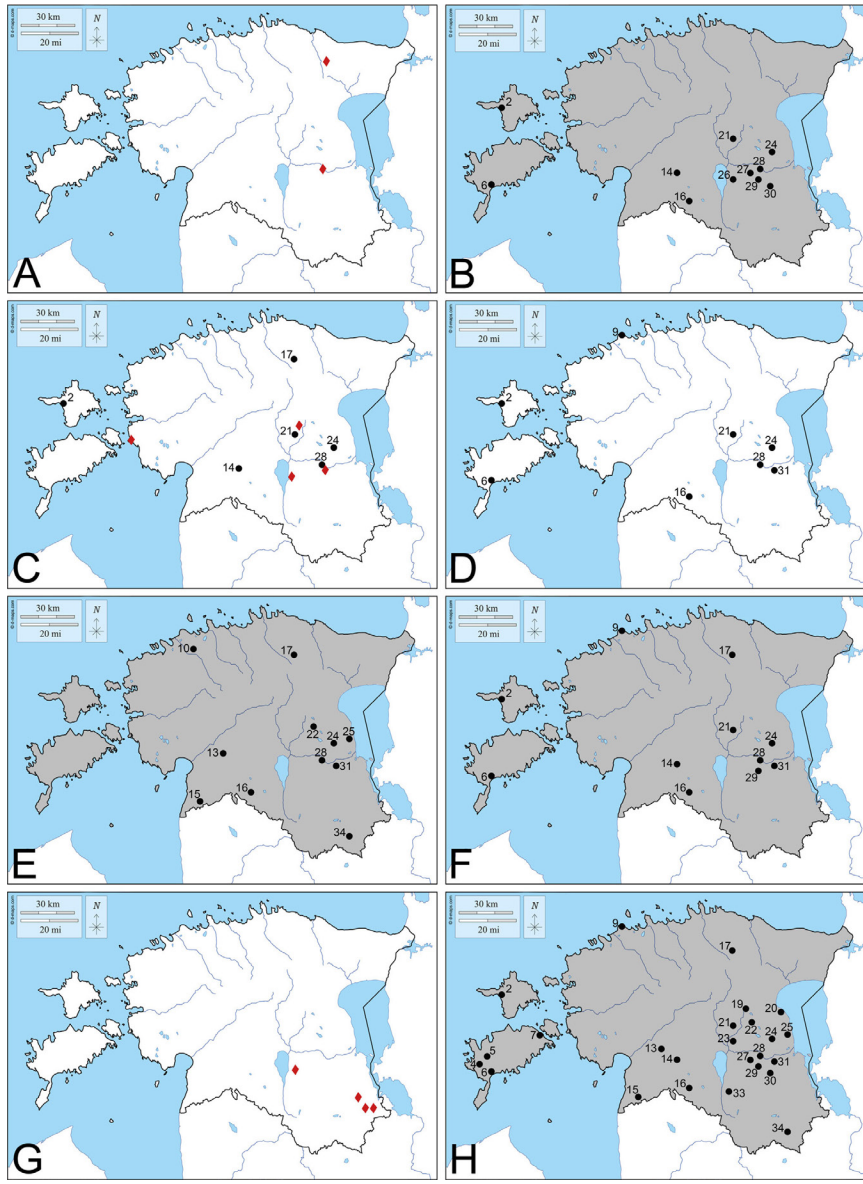

FIGURE 4. Maps showing the historic and current collection points of individual mosquito species in Estonia. **A**, *Aedes* (*Ochlerotatus*) *sticticus*; **B**, *Aedes* (*Ochlerotatus*) *riparius*; **C**, *Anopheles* (*Anopheles*) *algeriensis*; **D**, *Anopheles* (*Anopheles*) *claviger*; **E**, *Anopheles maculipennis* complex; **F**, *Anopheles* (*Anopheles*) *plumbeus*; **G**, *Coquillettidia* (*Coquillettidia*) *richiardii*; **H**, *Culex pipiens* complex.. For details, see Fig. 2.

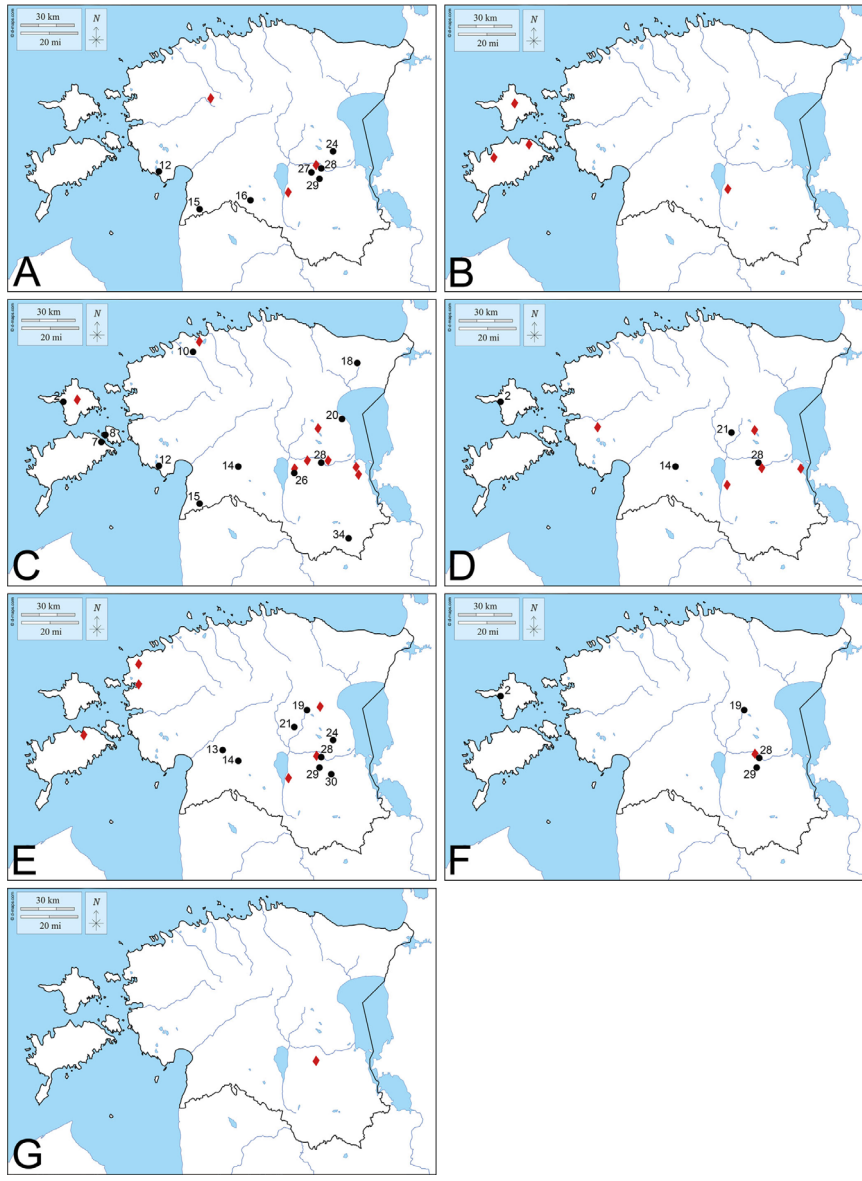

FIGURE 5. Maps showing the historic and current collection points of individual mosquito species in Estonia. **A**, *Culex* (*Neoculex*) *territans*; **B**, *Culiseta* (*Culicella*) *fumipennis*; **C**, *Culiseta* (*Culicella*) *morsitans*; **D**, *Culiseta* (*Culicella*) *ochroptera*; **E**, *Culiseta* (*Culiseta*) *alaskaensis*; **F**, *Culiseta* (*Culiseta*) *annulata*; **G**, *Culiseta* (*Culiseta*) *bergrothi*. For details, see Fig. 2, except for data for *Cs. bergrothi*, which is provided according to Khalin & Aibulatov (2020).

28. *Culex* (*Neoculex*) *territans* Walker, 1856
(Fig. 5A)

Published sources: Remm (1957: 157, as *Culex apicalis* Adams, 1903), Burtin (2014: 41), Kirik *et al*. (2021: 11).

Voucher material: 1♀, Tartu (58° 22′ 52″ N, 26° 42′ 49″ E), 08.IX.2016, H. Kirik leg., H. Kirik det., sweep net, IZBE0210230, GenBank: OK465180; 1♂, Tartu (58° 22′ 17″ N, 26° 41′ 58″ E), 20.IX.2016, H. Kirik leg., H. Kirik det., sweep net, IZBE0210250.

Comment: 50 mosquitoes (0.2% of all specimens collected) were identified as *Cx. territans*, making it the least common *Culex* species in Estonia. *Culex territans* was collected from July to October. This species is likely more common than the results of this fieldwork indicate, and their very low numbers are expected to be because of collection bias.

Genus *Culiseta* Felt, 1904

29. *Culiseta* (*Culicella*) *fumipennis* (Stephens, 1825) (Fig. 5B)

Published source: Remm (1957: 152, as *Theobaldia fumipennis*).

Voucher material: None.

Comment: Five females of *Cs. fumipennis* were previously reported from Estonia (Remm 1957). No individuals were found during this study. Of countries neighboring Estonia, *Cs. fumipennis* has been collected in Sweden (Lundström *et al.* 2013) and a province in northwestern Russia adjacent to Estonia (Khalin & Aibulatov 2020).

30. *Culiseta* (*Culicella*) *morsitans* (Theobald, 1901)
(Fig. 5C)

Published source: Remm (1957: 152, as *Theobaldia morsitans*).

Voucher material: 1♀, Soovere (58° 19′ 17″ N, 26° 40′ 56″ E), 09–12.VII.2020, H. Kirik leg., H. Kirik det., EVS trap, IZBE0210231; 1♀, Roosi (58° 23′ 25″ N, 26° 44′ 46″ E), 14–17.VII.2020, L. Tummeleht leg., H. Kirik det., Mosquito Magnet trap, IZBE0210232, GenBank: OK465181; 1♂, Maiorg (58° 16′ 41″ N, 26° 20′ 3″ E), 14–28.VI.2009, O. Kurina leg., H. Kirik det., Malaise trap, IZBE0210233.

Comment: 41 mosquitoes (0.2% of all specimens collected) were identified as *Cs. morsitans*, making it the most commonly collected *Culiseta* species during this study. *Culiseta morsitans* was found in insect traps from June to September. The low number of individuals collected is likely due to collection bias.

31. *Culiseta* (*Culicella*) *ochroptera* (Peus, 1935)
(Fig. 5D)

Published sources: Remm (1957: 152, as *Theobaldia ochroptera*), Kirik *et al*. (2021: 11).

Voucher material: 1♀, Tipu (58° 21′ 44″ N, 25° 3′ 44″ E), 22–26.VI.2018, H. Kirik leg., H. Kirik det., Mosquito Magnet trap, IZBE0210234, GenBank: OK465182; 1♀, Tartu (58° 23′ 05″ N, 26° 42′ 19″ E), 14.IX.2016, H. Kirik leg., H. Kirik det., sweep net, IZBE0210235, GenBank: OK465183; 1♀, Tartu (58° 21′ 23″ N, 26° 44′ 31″ E), 24.IX.2017, T. Kesküla leg., H. Kirik det., sweep net, IZBE0210236.

Comment: 11 mosquitoes were identified as *Cs. ochroptera*. These specimens were collected from August to October. The low number of individuals collected is likely to be due to sampling bias.

32. *Culiseta* (*Culiseta*) *alaskaensis* (Ludlow, 1906)
(Fig. 5E)

Published source: Remm (1957: 151, as *Theobaldia alaskaensis*).

Voucher material: 1♀, Undi (58° 29′ 44″ N, 26° 54′ 00″ E), 21–22.IV.2015, L. Tummeleht leg., H. Kirik det., Mosquito Magnet trap, IZBE0210237, GenBank: OK465184; 1♀, Ülenurme (58° 19′ 3″ N, 26° 43′ 23″ E), 11–14.VIII.2020, H. Kirik leg., H. Kirik det., Mosquito Magnet trap, IZBE0210238, GenBank: OK465185; 1♂, Kibuvitsa (58° 46′ 3″ N, 26° 30′ 46″ E), May to October 2020, L. Laaser leg., H. Kirik det., light trap, IZBE0210242.

Comment: 37 mosquitoes (0.2% of all specimens collected) were identified as *Cs. alaskaensis*, making it the second most common *Culiseta* species in Estonia based on this study. *Culiseta alaskaensis* appears to be active from April to August. The low number of individuals collected is likely to be due to sampling bias.

33. *Culiseta* (*Culiseta*) *annulata* (Schrank, 1776)
(Fig. 5F)

Published sources: Remm (1957: 151, as *Theobaldia annulata*), Burtin (2014: 42), Kirik *et al*. (2021: 11).

Voucher material: 1♀, Ülenurme (58° 19′ 3″ N, 26° 43′ 23″ E), 11–14.VIII.2020, H. Kirik leg., H. Kirik det., Mosquito Magnet trap, IZBE0210239, GenBank: OK465186; 1♀, Ülenurme (58° 19′ 3″ N, 26° 43′ 23″ E), 14–17.VIII.2020, H. Kirik leg., H. Kirik det., Mosquito Magnet trap, IZBE0210240, GenBank: OK465187; 1♂, Tartu (58° 21′ 23″ N, 26° 44′ 31″ E), 17.IX.2017, T. Kesküla leg., H. Kirik det., sweep net, IZBE0210246.

Comment: 28 mosquitoes (0.1% of all specimens collected) were identified as *Cs. annulata*. These mosquitoes were found throughout the warm season, from May to October, and seem to have the longest period of activity of all of the *Culiseta* species in Estonia. The low number of individuals collected is likely to be due to collection bias.

34. *Culiseta* (*Culiseta*) *bergrothi* (Edwards, 1921)
(Fig. 5G)

Published source: Khalin *et al*. (2020: 74).

Voucher material: None.

Comment: One female of *Cs. bergrothi* collected in southeastern Estonia is preserved in the Zoological Institute of the Russian Academy of Sciences (Khalin & Aibulatov 2020). This species was previously noted to be present in Estonia according to Fauna Europaea (Snow & Ramsdale 2014), but the origin of that record is unknown as no other records have been found by the authors. *Culiseta bergrothi* has also been reported from provinces in northwestern Russia adjacent to Estonia (Khalin & Aibulatov 2020), Finland (Culverwell 2018; Culverwell *et al*. 2021) and Sweden (Lundström *et al.* 2013).

Notes on species not included in the list above

Genus *Aedes* Meigen, 1818

***Aedes* (*Aedes*) *geminus* Peus, 1970**

Published sources: None.

Voucher material: None.

Comment: *Aedes geminus* has been reported from Lithuania (Pakalniškis *et al.* 2000; Bernotienė & Lučiūnaitė 2011), a province in northwestern Russia adjacent to Estonia (Khalin & Aibulatov 2020), Finland (Culverwell 2018; Culverwell *et al*. 2021) and Sweden (Lundström *et al.* 2013). Therefore, it may also be present in Estonia, but more work is needed to verify this, as the adult females of this species are difficult to distinguish from those of *Ae. cinereus*.

***Aedes* (*Dahliana*) *geniculatus* (Olivier, 1791)**

Published sources: None.

Voucher material: None.

Comment: *Aedes geniculatus* has been reported from Lithuania (Pakalniškis *et al.* 2000), provinces in northwestern Russia adjacent to Estonia (Khalin & Aibulatov 2020), Finland (Culverwell 2018; Culverwell *et al*. 2021) and Sweden (Lundström *et al.* 2013), and could be present in Estonia. This species probably has not been collected during fieldwork due to collection bias.

***Aedes* (*Ochlerotatus*) *euedes* Howard, Dyar & Knab, 1913**

Published sources: None.

Voucher material: None.

Comment: *Ae*des *euedes* is a member of the *Ae. annulipes* group. It reported from Lithuania (Pakalniškis *et al.* 2000), Latvia (Spungis 2000), provinces in northwestern Russia adjacent to Estonia (Khalin & Aibulatov 2020), Finland (Culverwell 2018; Culverwell *et al*. 2021) and Sweden (Lundström *et al.* 2013). While no specimens have been reported from Estonia thus far, the species is likely to be present but overlooked, especially as this species appears to be much less common in the Nordic-Baltic region than *Ae*. *annulipes* and *Ae*. *cantans*, which also belong to the *Ae. annulipes* group (Khalin & Aibulatov 2020; Lundström *et al.* 2013).

***Aedes* (*Ochlerotatus*) *pullatus* (Coquillett, 1904)**

Published source: Kirik *et al*. (2021: 11).

Voucher material: None.

Comment: Mosquitoes with morphological characteristics most similar to *Ae. pullatus* were found during the study, but DNA barcoding identified them as *Ae. communis*. These specimens had scales on the hypostigmal area of the thorax, which is unusual for *Ae. communis*. *Aedes pullatus* has been previously reported from Lithuania (Pakalniškis *et al.* 2000), provinces in northwestern Russia adjacent to Estonia (Khalin & Aibulatov 2020), Finland (Culverwell 2018; Culverwell *et al*. 2021) and Sweden (Lundström *et al.* 2013). However, the occurrence of this species in Estonia remains uncertain.

***Aedes* (*Ochlerotatus*) *punctodes* Dyar, 1922**

Published sources: None.

Voucher material: None.

Comment: Females of *Ae. punctodes* are difficult to distinguish from the females of *Ae. punctor*. This species has been reported from Finland (Culverwell 2021) and Sweden (Lundström *et al.* 2013), and could also be present in Estonia, but this requires further research.

***Aedes* (*Rusticoidus*) *rusticus* (Rossi, 1790)**

Published sources: None.

Voucher material: None.

Comment: *Aedes rusticus* has been recognized as a species in Estonia in several mosquito checklist (Khalin & Aibulatov 2020; Snow & Ramsdale 1999; Robert *et al.* 2019). However, as was pointed out by Huldén & Huldén (2014) *Ae. rusticus* has been referred to historically by the synonym *Ae. maculatus*, but that name has also been mistakenly applied to *Ae. cantans*. In fact, the first checklist of mosquitoes in Estonia by H. Remm also included *Ae. maculatus*, but the number of specimens collected, as well as the description of their bionomics indicates that the species is *Ae. cantans*, not *Ae. rusticus*. This misunderstanding may be the reason why *Ae. rusticus* has been

reported to be present in Estonia, although no verified specimens have been collected in the country. However, *Ae. rusticus* has also been thought to be present in Lithuania (Pakalniškis *et al.* 2000) and Latvia (Spungis 2000), and has been reported from Sweden (Lundström *et al.* 2013). Therefore, this species could also be present in Estonia.

Genus *Anopheles* Meigen, 1818

***Anopheles daciae* Linton, Nicolescu & Harbach, 2004** (in Nicolescu *et al*. 2004)

Published sources: None.

Voucher material: None.

Comment: It can be difficult to distinguish *An. daciae* from *An. messeae* based on morphology. Nucleotide polymorphisms in ITS2 sequences are currently the best way to distinguish these two species, but this was not done in this study. *Anopheles daciae* has been reported from Finland (Culverwell *et al.* 2020; Culverwell *et al*. 2021), and could also be present in Estonia.

Discussion

This is the first comprehensive update to the mosquito fauna of Estonia since the publication of the original checklist by Remm (1957). The new checklist was compiled based on 24,344 adult mosquitoes (94.2% females) collected from 2008 to 2020, while also considering historic records and information from neighboring countries. Regrettably, mosquitoes collected by Remm, and used to compile the first checklist, have not been preserved and could not be verified. In total, the contemporary list includes 34 species, 27 of which were confirmed with voucher specimens; however, no specimens were collected to confirm the presence of seven other species in the country. All in all, additional collection efforts are required for a more thorough and detailed overview of the local mosquito fauna.

The updated checklist includes numerous changes compared to the historic list, which featured 30 species based on 12,204 mosquitoes (Remm 1957). Most importantly, four species were added to the list: *An. messeae*, *Ae. hexodontus*, *Ae. sticticus* and *Cs. bergrothi*. While the inclusion of the first three species is backed by numerous recently collected specimens, *Cs. bergrothi* is included based on a single specimen from Estonia stored at the Zoological Institute of the Russian Academy of Sciences (Khalin & Aibulatov 2020). The possible occurrence of seven additional species, *An. daciae, Ae. geminus, Ae. geniculatus*, *Ae. euedes*, *Ae. pullatus*, *Ae. punctodes* and *Ae. rusticus*, was discussed. Those species are present in neighboring countries, but could not be included in the updated list without evidence to verify their presence in Estonia. Interestingly, when comparing the original checklist with the present one, the same four species have remained the most numerous, making up the majority of the specimens collected in both cases. *Aedes communis* remains the most common mosquito in Estonia, as it made up 29.7% of all specimens collected in 1957 and 30.1% of all mosquitoes collected between 2008 and 2020 in the present study. *Aedes communis* is followed by *Ae. punctor*, *Ae. cataphylla* and mosquitoes of the *Ae. annulipes* group. It is important to note that all four species are most active during late spring or early summer: the first three are especially numerous during May, while members of the *Ae. annulipes* group tend peak in June. Naturally, there were also numerous differences in the abundance of various species between the two checklists, but it is unknown whether these were due to genuine change or merely because of differences in collection methods and study sites.

Several mosquito species are likely underrepresented in this study due to the chosen collection sites and methods of collection. In fact, the relatively low numbers of many *Aedes*, *Anopheles*, *Culex* and *Culiseta* species collected in this study are likely due to collection bias and further work is needed to understand their true abundance in Estonia. Also, study sites were mostly concentrated in southeastern Estonia, covering the areas of the eastern Lowlands and Drumlins, as well as the southern Uplands (Villoslada *et al.* 2017). Islands and the coast of the mainland were also covered, but require more long-term collecting effort to better understand how brackish water affects the local mosquito fauna. For example, it is clear that *Ae. caspius* is common in these areas, but other salinity tolerant species, for example *Ae. dorsalis*, require further research. It is likely that the makeup and bionomics of the costal mosquito fauna are markedly different from areas on the mainland. Collection sites of the current study generally mimicked the locations reported by Remm (1957). However, the central area of the mainland, including parts of the Central Estonian Plain, as well as the Pandivere Uplands and the Northern Plain (Villoslada *et al.* 2017), received little attention in both cases. It is also important to note that this research was based solely on active adult mosquitoes, the

vast majority of which were females due to the chosen collection methods. In future, more work should be done to collect overwintering adults, which would allow for a more efficient collection of mosquito species that may not be attracted to baited traps. Moreover, collections larvae would not only improve the checklist, but would also provide additional information about the ecology of the species. It would likewise be beneficial to collect more male mosquitoes. While males do not require blood meals and are thus far less studied, they provide additional verification of the occurrence of some species. For example, the presence of *Ae. diantaeus*, which is morphologically similar to both *Ae. intrudens* and *Ae. communis*, was finally verified in Estonia based on a male specimen. Also, many female mosquitoes of the *Ae. annulipes* group can be difficult to identify due to overlapping morphological characteristics, as well as inconclusive results of DNA barcoding. However, it is relatively easy to distinguish the males of these species based on structures of their genitalia.

An updated checklist allows for a better understanding of the mosquito-borne pathogens circulating among local dipterans. For example, tularemia is a disease caused by the bacterium *Francisella tularensis* (McCoy & Chapin), which occurs throughout the northern hemisphere. It manifests in humans with influenza-like symptoms and numerous other ailments, based on the route of infection (Maurin & Gyuranecz 2016). *Francisella tularensis* is normally confined to animals on a few islands of Estonia, but one or two human infections occur in the country almost every year (Health Board 2012, 2016, 2020). Although mosquitoes are one of several arthropods capable of transmitting the bacterium, natural infections have been detected in *Ae. cinereus*, *Ae. communis*, *Ae. punctor*, *Ae. sticticus*, *Ae. vexans* and *Cx. pipiens* (Lundström *et al.* 2011; Dryselius *et al.* 2019). Furthermore, the filarial nematode *Dirofilaria repens* Railliet & Henry appears to also have become established in Estonia, since it has been found in local dogs several times since 2008 (Jokelainen *et al.* 2016). The mosquito-borne *Dirofilaria repens* normally parasitizes subcutaneous tissues of carnivores and is often asymptomatic in dogs, but can also infect humans, resulting in skin nodules, ocular dirofilariasis or other complications (Capelli *et al.* 2018; Ciuca *et al.* 2020; Pupić-Bakrač *et al.* 2021). In fact, autochthonous human cases have already been reported in countries neighboring Estonia (Melbarde-Gorkusa *et al.* 2011; Pietikäinen *et al.* 2017). Thus far, numerous species belonging to the genera *Aedes*, *Anopheles*, *Coquillettidia* and *Culex* have been indicated in carrying *Dirofilaria repens*, as reported by Kronefeld *et al.* (2014), Kemenesi *et al.* (2015), Şuleşco *et al.* (2016) and Shaikevich *et al.* (2019a). Importantly, Shaikevich *et al*. (2019a) found that *Ae. communis* could be one of the species effective in spreading *Dirofilaria* species in Russia. Additionally, there are also some mosquito-borne viruses circulating in northern Europe (Francy *et al.* 1989; Barzon 2018). For example, Sindbis virus, which is carried long distances by migrating birds and transmitted to humans by mosquitoes, is especially noteworthy in the Nordic countries (Kurkela *et al.* 2005; Bergqvist *et al.* 2015), but the virus has also been isolated from birds in Estonia (Uryvaev *et al.* 1992). Generally, ornithophilic species like *Cx. pipiens*, *Cx. torrentium* and *Cs. morsitans* are thought to be important carriers of the Sindbis virus (Francy *et al.* 1989). Based on this information, *Ae. communis*, which is overall the most numerous mosquito in Estonia, and *Cx. pipiens*/*torrentium*, which are especially active at the end of summer, are the most likely species to become important vectors in the country.

There are still notable caps in our knowledge of mosquito diversity in Estonia, as biting dipterans were largely ignored during the latter half of the last century and the country still lacks a continuous mosquito monitoring program. Furthermore, scenarios of climate change predict that the annual mean temperature is likely to increase by 2.3–4.5°C in Estonia by the year 2100, and during the same time the average yearly precipitation could increase anywhere between 4–46% (Kont *et al.* 2003). This will likely influence the length of time suitable for mosquito development, as well as the availability of larval habitats in the country. Also, it is well known that alterations in land use, international trade and travel have led to changes in the diversity and distribution of various arthropods, including many mosquitoes (Brugueras *et al.* 2020; Medlock *et al.* 2012; Rochlin *et al.* 2016; Brugueras *et al.* 2020). Hence, there is a clear need for further studies on both blood-sucking dipterans and insect-borne pathogens in Estonia. Extra attention should be paid to the international airport and large harbors, which can act as entry points for non-native mosquitoes (Sukehiro *et al.* 2013; Ibáñez-Justicia *et al.* 2020). Furthermore, mosquito collection activities should be more evenly spread out in Estonia to sample as many biotypes as possible. Finally, insect-borne pathogens require more attention. For example, how important mosquitoes are in transmitting *Francisella tularensis* in the region and which species carry *Dirofilaria repens* in Estonia remains to be investigated.

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Kirik, H., Burtin, V., Tummeleht, L. & Kurina, O. (2021) Friends in all the green spaces: weather dependent changes in urban mosquito (Diptera: Culicidae) abundance and diversity. *Insects*, 12 (4), 352.
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CURRICULUM VITAE

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Education:

2015-2022	Estonian University of Life Sciences, PhD studies in Environmental Sciences and Applied Biology
2012-2015	University of Tartu, MSc studies in Biology
2009-2012	University of Tartu, BSc studies in Ecology and Biodiversity Conservation
1997-2009	August Kitzberg Gymnasium

Professional employment:

2021	Estonian University of Life Sciences, Chair of Biodiversity and Nature Tourism, Specialist
2018-2021	Estonian University of Life Sciences, Chair of Biodiversity and Nature Tourism, Junior researcher
2016-2017	Estonian University of Life Sciences, Institute of Veterinary Medicine and Animal Sciences, Specialist

Languages: Estonian – native tongue; English – very good

Research interests:

Medical Entomology, Molecular Phylogenetics, Systematics, Ecology

Professional training:

2018	Lecture "Epidemiology and Surveillance of Communicable Diseases" at the University of Tartu in Estonia
2017	Course "Molecular Biology in Animal Sciences" organized by the Estonian University of Life Sciences in Estonia

2017	NOVA course "Molecular Epidemiology of Infectious Diseases" organized by the University of Helsinki in Finland
2017	Training school "Phylogenetic and Population Genetic Tools for Vectors and Vector-Borne Pathogen" organized by Institute of the Hygiene and Tropical Medicine in Portugal
2017	Online course "Medical Entomology" organized by the Pasteur Institute
2016	Two-Day Field Training Course on Vector Control organized by the European Mosquito Control Association in Portugal

Participation in projects:

2017-2022	L170171PKZO "Developing biological and technical solutions for reducing the number of hematophagous insects in the vicinity of farm animals"
2016-2017	8P160014VLVP "Molecular epidemiology of pathogenic protozoa as well as the metagenomics of vector-borne pathogens and vector ecology"
2014-2019	IUT21-1 "Non-additive impact of border-richness to biota"

ELULOOKIRJELDUS

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Haridustee:

2015-2022	Eesti Maaülikool, doktorantuur keskkonnateaduse ja rakendusbioloogia erialal
2012-2015	Tartu Ülikool, magistrantuur bioloogia erialal
2009-2012	Tartu Ülikool, bakalaureus ökoloogia ning elustiku kaitse erialal
1997-2009	August Kitzbergi nimeline Gümnaasium

Teenistuskäik:

2021	Eesti Maaülikool, Elurikkuse ja loodusturismi õppetool, Spetsialist
2018-2021	Eesti Maaülikool, Elurikkuse ja loodusturismi õppetool, Nooremteadur
2016-2017	Eesti Maaülikool, Veterinaarmeditsiini ja loomakasvatuse instituut, Spetsialist

Keelteoskus: Eesti keel – emakeel; inglise keel – väga hea

Teadustöö põhisuunad:

Meditsiiniline entomoloogia, molekulaarne fülogeneetika, süstemaatika, ökoloogia

Enesetäiendus ja koolitused:

2018	Õppeaine "Nakkushaiguste epidemioloogia ja seire" Tartu Ülikoolis
2017	Kursus "Molekulaarne bioloogia loomateaduses" Eesti Maaülikoolis
2017	Kursus "Nakkushaiguste molekulaarne epidemioloogia" Soomes Helsingi Ülikoolis

2017	Kursus "Fülogeneetilised ja populatsioonigeneetilised meetodid vektoritele ja vektorhaigustele" Portugalis Hügieeni ja Troopilise Meditsiini Instituudis
2017	Pasteuri Instituudi veebikursus "Meditsiiniline entomoloogia"
2016	EMCA kahepäevane vektorite kontrolli välitööde kursus Portugalis

Uurimisprojektides osalemine:

2017-2022	L170171PKZO "Bioloogiliste ja tehniliste lahenduste välja töötamine vereimejate putukate arvukuse vähendamiseks karjatatavate loomade ümbruses"
2016-2017	8P160014VLVP "Eestis vähe uuritud algloomtõbede molekulaarepidemioloogia ning putuksiirutajatega levivate nakkuste metagenoomika ja siirutajate ökoloogia"
2014-2019	IUT21-1 "Piiririkkuse mitteaditiivne mõju elustikule"

LIST OF PUBLICATIONS

1.1. Articles indexed by Thomson Reuters Web of Science

Kirik, H.; Tummeleht, L.; Kurina, O. (2022). Rediscovering the mosquito fauna (Diptera: Culicidae) of Estonia: an annotated checklist with distribution maps and DNA evidence. *Zootaxa*, 5094(2), 261-287. DOI: 10.11646/zootaxa.5094.2.3

Herm, R.; **Kirik, H.**; Vilem, A.; Zani, L.; Forth, J.H.; Müller, A.; Michelitsch, A.; Wernike, K.; Werner, D.; Tummeleht, L.; Kampen, H.; Viltrop, A. (2021). No evidence for African swine fever virus DNA in haematophagous arthropods collected at wild boar baiting sites in Estonia. *Transboundary and Emerging Diseases*, 68(5), 2696-2702. DOI: 10.1111/tbed.14013

Kirik, H.; Burtin, V.; Tummeleht, L.; Kurina, O. (2021). Friends in all the green spaces: weather dependent changes in urban mosquito (Diptera: Culicidae) abundance and diversity. *Insects*, 12(4), 352. DOI: 10.3390/insects12040352

Kurina, O.; **Kirik, H.** (2021). Every single specimen counts: a new *Docosia* Winnertz (Diptera: Mycetophilidae) species described from a singleton. *Insects*, 12(12), 1069. DOI: 10.3390/insects12121069

Tummeleht, L.; Jürison, M.; Kurina, O.; **Kirik, H.**; Jeremejeva, J.; Viltrop, A. (2020). Diversity of Diptera species in Estonian pig farms. *Veterinary Sciences*, 7(1), 13. DOI: 10.3390/vetsci7010013

Kirik, H.; Tummeleht, L.; Lilja, T.; Kurina, O. (2020). Novel Mitochondrial DNA Lineage Found among *Ochlerotatus communis* (De Geer, 1776) of the Nordic-Baltic Region. *Insects*, 11(6), 397. DOI: 10.3390/insects11060397

Kurina, O.; **Kirik, H.**; Õunap, H.; Õunap, E. (2019). The northernmost record of a blood-sucking ectoparasite, *Lipoptena fortisetosa* Maa (Diptera: Hippoboscidae), in Estonia. *Biodiversity Data Journal*, 7, e47857. DOI: 10.3897/BDJ.7.e47857

Lilja, T.; Troell, K.; **Kirik, H.**; Lindstrom, A. (2018). A distinct group of north European *Aedes vexans* as determined by mitochondrial and nuclear markers. *Medical and Veterinary Entomology*, 32(3), 282−289. DOI: 10.1111/mve.12294

5.2. Conference abstracts

Kirik, H.; Tummeleht, L.; Lilja, T.; Kurina, O. (2018). Species delimitation in the *Ochlerotatus communis* (De Geer) complex (Culicidae), a novel mitochondrial DNA (mtDNA) line from Europe. 9th International Congress of Dipterology. Abstract Volume: 9th International Congress of Dipterology, 25-30 November 2018, Windhoek, Namibia. Ed. A. H. Kirk-Spriggs; B. S. Muller. International Congress of Dipterology, 132

6.3. Popular science articles

Tikk, M.; **Kirik, H.** (2020). Loodus- ja tervishoidlik sääse-, puugi- ja parmutõrje. Eesti Loodus, 71 (6), 66−68.

Kurina, O.; Kull, T.; **Kirik, H.** (2016). Sääsk, kes ründas Karl Ernst von Baeri. Eesti Loodus, 67 (4), 70−71

VIIS VIIMAST KAITSMIST

LIINA JÜRISOO
DISTRIBUTION OF AND DAMAGES CAUSED BY DUTCH ELM DISEASE AGENTS IN NORTHERN EUROPE
JALAKASURMA LEVIK JA KAHJUSTUSED PÕHJAEUROOPAS
Professor Rein Drenkhan
11. november 2021

HEIKI LILL
NOVEL APPLICATION PRINCIPLES FOR ENERGY STORAGE TECHNOLOGIES IN NEARLY ZERO ENERGY BUILDINGS
ERINEVATE ENERGIA SALVESTUSTEHNOLOOGIATE UUDSED RAKENDUSPÕHIMÕTTED LIGINULLENERGIAHOONETES
Teadur Alo Allik, professor Andres Annuk
24. november 2021

PIRET RAUDSEPP
POLYPHENOLIC COMPOSITION OF RHUBARB (*RHEUM RHAPONTICUM* L.) AND BLACKCURRANT (*RIBES NIGRUM* L.), ANTIBACTERIAL AND FREE RADICAL SCAVENGING PROPERTIES OF THESE PLANTS IN COMPARISON WITH SOME OTHER FOOD PLANTS
HARILIKU RABARBERI (*RHEUM RHAPONTICUM* L.) JA MUSTA SÕSTRA (*RIBES NIGRUM* L.) POLÜFENOOLNE KOOSTIS, NENDE TAIMEDE ANTIBAKTERIAALSE TOIME JA VABADE RADIKAALIDE SIDUMISE VÕIME VÕRDLUS MÕNEDE TEISTE TOIDUTAIMEDEGA
Professor Tõnu Püssa, vanemteadur Ave Kikas
10. detsember 2021

INGRID BENDER
EFFECT OF ORGANIC MANAGEMENT METHODS ON YIELD AND QUALITY OF CARROT AND ON WEEDS
MAHEVILJELUSE MEETODITE MÕJU PORGANDI SAAGILE JA KVALITEEDILE NING UMBROHTUDELE
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