Clearing up *Culex* Confusion

*A Basis for Virus Vector Discrimination in Europe*

JENNY C. HESSON
Mosquito species of the *Culex* genus are the enzootic vectors for several bird-associated viruses that cause disease in humans. In Europe, these viruses include Sindbis (SINV), West Nile and Usutu viruses. The morphologically similar females of *Cx. torrentium* and *Cx. pipiens* are potential vectors of these viruses, but difficulties in correctly identifying the mosquito species have caused confusion regarding their respective distribution, abundance, ecology, and consequently their importance as vectors. Species-specific knowledge from correctly identified field material is however of crucial importance since previous research shows that the relatively unknown *Cx. torrentium* is a far more efficient SINV vector than the widely recognized *Cx. pipiens*. The latter is involved in the transmission of several other viruses, but its potential importance for SINV transmission is debated.

In this thesis I describe the development of a molecular method for species identification, based on reliably identified males of *Cx. torrentium* and *Cx. pipiens*. This identification method was then used in consecutive studies on the distribution and relative abundance of the two species in Sweden and 12 other European countries, SINV field infection rates in mosquitoes identified to species level, and evaluation of potential trap bias associated with common sampling techniques.

The results showed that *Cx. torrentium* is a far more common species in Europe than previously assumed. In Sweden and Finland, it is the dominant species, accounting for 89% of the sampled *Culex* population. In central Europe, it is equally common to *Cx. pipiens*, while *Cx. pipiens* dominates south of the Alps Mountain range. Larvae of both species are often found together in both artificial containers (e.g. car tires) and natural sites. Also, a trapping bias against *Cx. torrentium* was revealed for CDC-traps. For the first time, SINV was isolated from species-identified *Cx. torrentium* and *Cx. pipiens* mosquitoes caught in the field, with *Cx. torrentium* being superior in infection rates (36/1,000 vs. 8.2/1,000). Future studies on SINV, as well as other mosquito-borne bird viruses in Europe, can hopefully gain from the baseline information provided here, and from principles of vector discrimination discussed in the thesis.

Keywords: *Culex torrentium*, *Culex pipiens*, mosquitoes, vector, ornithophilic, Sindbis virus, West Nile virus

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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## Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ae.</td>
<td><em>Aedes</em></td>
</tr>
<tr>
<td>CDC</td>
<td>Centers of Disease Control and Prevention</td>
</tr>
<tr>
<td>COI</td>
<td>Cytochrome oxidase I</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
</tr>
<tr>
<td>Cs.</td>
<td><em>Culiseta</em></td>
</tr>
<tr>
<td>Cx.</td>
<td><em>Culex</em></td>
</tr>
<tr>
<td>IR</td>
<td>Infection rate</td>
</tr>
<tr>
<td>MIR</td>
<td>Minimum infection rate</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PFU/mL</td>
<td>Plaque forming units per milliliter</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse Transcription-qPCR</td>
</tr>
<tr>
<td>SINV</td>
<td>Sindbis virus</td>
</tr>
<tr>
<td>SLEV</td>
<td>St. Louis encephalitis virus</td>
</tr>
<tr>
<td>USUV</td>
<td>Usutu virus</td>
</tr>
<tr>
<td>WEEV</td>
<td>Western equine encephalitis virus</td>
</tr>
<tr>
<td>WNV</td>
<td>West Nile virus</td>
</tr>
</tbody>
</table>
Introduction

Mosquito-borne viruses
Mosquitoes can transmit a wide range of human pathogens, including viruses, bacteria, nematodes and protozoans. For viruses alone, approximately 100 different species can infect humans and several others infect livestock and wild animals (Mullen and Durden 2009). All but a few mosquito-borne viruses belong to either of three different virus families: Togaviridae, Flaviviridae and Bunyaviridae, and cause infections ranging from asymptomatic to fever and arthritis, neurological symptoms and hemorrhagic fevers (Table 1) (Gould et al 2010).

The mosquito’s role as a vector is to transport the virus between two vertebrate individuals. Normally the virus transfer takes place between individuals belonging to the same or related species, i.e. the natural reservoir host population, which for mosquito-borne viruses are either mammals or birds (Mullen and Durden 2009). The transfer of virus to other species than the reservoir host, e.g. from birds to humans, is usually accidental and the virus cannot spread any further, hence the name dead-end, incidental or tangential host. These hosts do not produce high enough viremias to further infect a blood-feeding mosquito.

Viruses that mainly infect other vertebrate animals but occasionally infect humans are also called zoonotic, such as Sindbis virus (SINV) and West Nile virus (WNV). Some viruses however are not dependent on non-human hosts but are mainly hosted and maintained by humans, such as Dengue virus.

Mosquito-borne bird viruses
Mosquito-borne viruses that are hosted by birds belong to either of two genera: alphavirus (family Togaviridae) and flavivirus (family Flaviviridae). Although belonging to different virus families most of these viruses demonstrate a very similar ecology, involving the same or closely related mosquito vector species and bird hosts.

The alphaviruses that are found in the New World cause neurological symptoms such as encephalitis while the Old World alphaviruses cause arthritis, often following fever and rash (Suhrbier et al. 2012). The flaviviruses generally cause neurological diseases such as meningoencephalomyelitis (Gould and Solomon 2008). Many of these viruses commonly cause disease
not only in humans, but also in horses, that often show neurological symptoms (Gould et al. 2010).

Sindbis virus epidemiology

In this thesis, the focus lies on the transmission ecology of SINV (Togaviridae: alphavirus). SINV is a mosquito-born bird virus (Figure 1) that was first isolated in 1952 in Sindbis village, 30 km south of Cairo, Egypt, and has since then been found all over the Old World. SINV has an enveloped single stranded positive-sense RNA genome, with approximately 11,700 nucleotides. Genetically, it can be divided into five genotypes with representatives in Europe and South Africa (SINV-I), Australia (SINV-II), East Asia (SINV-III), Azerbaijan and China (SINV-IV) and New Zealand (SINV-V), based on analysis of 340 nucleotides of the nuclear Envelope 2 glycoprotein gene (Lundström and Pfeffer 2010). All human infections are from the SINV-I genotype, and cases have been reported from Sweden (Ockelbo disease), Finland (Pogosta), western Russia (Karelian fever) and South Africa (Sindbis fever) (Jupp 2001). Although the disease has many different names, the symptoms are the same, including rash, fever and long lasting arthritis (Kurkela et al. 2005). Infections in horses, with neurological symptoms, have also been reported from South Africa (Mariëtjie Venter pers. com.).

![Figure 1. General transmission cycle of Sindbis virus in Sweden](image-url)
Most European cases of SINV infection (Figure 2) are reported in late summer from Finland, and mainly from the north Karelian region bordering to Russia (Sane et al. 2010, Brummer-Korvenkontio et al. 2002, National Institute for Health and Welfare 2014). The seroprevalence in this region is >9% (Kurkela et al. 2008). In Sweden, usually only a couple of cases are reported each year (Lundström et al, 1991, Lundström et al. 2014), mainly occurring in the provinces of Hälsingland, Gästrikland and Dalarna where the seroprevalence is around 3.6% (Lundström et al. 1991). However, in the fall of 2013 there was an unusually large outbreak involving over 60 diagnosed cases in the small town Lövånger in Västerbotten, far north of the normal epidemic area (Ström 2013). The overall seroprevalence in this part of Sweden is 1.0–2.9% (Lundström et al. 1991, Ahlm et al. 2014).

It has been proposed that SINV causes outbreaks every 7th year in northern Europe (Brummer-Korvenkontio et al. 2002), and calculations of the average annual incidence of SINV infections per 100 000 citizens in Finland, in 1995 to 2003, showed increased incidence in the outbreak years 1995 and 2003 (Kurkela et al. 2008). However, data on human infections in both Sweden and Finland only show a weak pattern and the reliability is very low since many factors influence the number of cases reported. In Finland, SINV is a notifiable disease since 1995, whereas in Sweden it is not. A better understanding of the potential temporal variations could potentially be reached by looking directly at the vector population that transmits the virus (Lundström et al. 2014).

In South Africa, antibodies to SINV are found in humans all over the country, but transmission occurs mainly across the inland plateau (Storm et al. 2014). In this area, human cases of rash, fever, and arthralgia occur sporadically every summer and larger outbreaks, involving several hundreds of cases, occurred in 1974 and 1984 (McIntosh et al. 1976, Storm et al. 2014).
Table 1. Mosquito-borne viruses that cause symptomatic disease in humans.

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
<th>Complex</th>
<th>Virus</th>
<th>Host</th>
<th>Distribution</th>
<th>Disease</th>
<th>Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>Togaviridae</td>
<td>Alpha</td>
<td>BF</td>
<td>Barmah forest (BFV)</td>
<td>M</td>
<td>Aus</td>
<td>A</td>
<td>Culex</td>
</tr>
<tr>
<td>Togaviridae</td>
<td>Alpha</td>
<td>Semliki forest (SF)</td>
<td>Ross river (RRV)</td>
<td>M</td>
<td>Aus</td>
<td>A</td>
<td>Culex</td>
</tr>
<tr>
<td>Togaviridae</td>
<td>Alpha</td>
<td>SF</td>
<td>O'nyong-nyong (ONNV)</td>
<td>U</td>
<td>A</td>
<td>A</td>
<td>Anopheles</td>
</tr>
<tr>
<td>Togaviridae</td>
<td>Alpha</td>
<td>SF</td>
<td>Chikungunya (CHIKV)</td>
<td>M</td>
<td>A, As, E</td>
<td>A</td>
<td>Aedes</td>
</tr>
<tr>
<td>Togaviridae</td>
<td>Alpha</td>
<td>SF</td>
<td>Mayaro (MAYV)</td>
<td>M</td>
<td>SA</td>
<td>A</td>
<td>Haemagogus</td>
</tr>
<tr>
<td>Togaviridae</td>
<td>Alpha</td>
<td>VEE</td>
<td>Venezuelan equine encephalitis (VEEV)</td>
<td>M</td>
<td>SA</td>
<td>N</td>
<td>Culex</td>
</tr>
<tr>
<td>Togaviridae</td>
<td>Alpha</td>
<td>EEE</td>
<td>Eastern equine encephalitis (EEEV)</td>
<td>B</td>
<td>NA</td>
<td>N</td>
<td>Culiseta</td>
</tr>
<tr>
<td>Togaviridae</td>
<td>Alpha</td>
<td>WEE</td>
<td>Western equine encephalitis (WEEV)</td>
<td>B</td>
<td>NA</td>
<td>N</td>
<td>Culex</td>
</tr>
<tr>
<td>Togaviridae</td>
<td>Alpha</td>
<td>WEE</td>
<td>Sindbis (SINV)</td>
<td>B</td>
<td>E, A</td>
<td>A</td>
<td>Culex</td>
</tr>
<tr>
<td>Flaviviridae</td>
<td>Flavi</td>
<td>JE</td>
<td>Kunjin (KUNV)</td>
<td>B</td>
<td>Aus</td>
<td>N</td>
<td>Culex</td>
</tr>
<tr>
<td>Flaviviridae</td>
<td>Flavi</td>
<td>JE</td>
<td>Murray Valley encephalitis (MVEV)</td>
<td>B</td>
<td>Aus</td>
<td>N</td>
<td>Culex</td>
</tr>
<tr>
<td>Flaviviridae</td>
<td>Flavi</td>
<td>JE</td>
<td>Japanese encephalitis (JEV)</td>
<td>B</td>
<td>As</td>
<td>N</td>
<td>Culex</td>
</tr>
<tr>
<td>Flaviviridae</td>
<td>Flavi</td>
<td>JE</td>
<td>St. Louis encephalitis (SLEV)</td>
<td>B</td>
<td>NA</td>
<td>N</td>
<td>Culex</td>
</tr>
<tr>
<td>Flaviviridae</td>
<td>Flavi</td>
<td>Ntaya</td>
<td>Rocio (ROCV)</td>
<td>B</td>
<td>SA</td>
<td>N</td>
<td>Culex</td>
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<td>Flaviviridae</td>
<td>Flavi</td>
<td>JE</td>
<td>Usutu (USUV)</td>
<td>B</td>
<td>E, A</td>
<td>N</td>
<td>Culex</td>
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<td>Flaviviridae</td>
<td>Flavi</td>
<td>JE</td>
<td>West Nile (WNV)</td>
<td>B</td>
<td>A, As, E, NA, SA</td>
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<td>Culex</td>
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<tr>
<td>Flaviviridae</td>
<td>Flavi</td>
<td>DEN</td>
<td>Dengue (DENV)</td>
<td>H</td>
<td>A, As, Aus, E, NA, SA</td>
<td>H</td>
<td>Aedes</td>
</tr>
<tr>
<td>Flaviviridae</td>
<td>Flavi</td>
<td>ZIKV</td>
<td>Zika (ZIKV)</td>
<td>H</td>
<td>A, As</td>
<td>A</td>
<td>Aedes</td>
</tr>
<tr>
<td>Flaviviridae</td>
<td>Flavi</td>
<td>YF</td>
<td>Yellow Fever (YFV)</td>
<td>H</td>
<td>A</td>
<td>H</td>
<td>Aedes</td>
</tr>
<tr>
<td>Bunyaviridae</td>
<td>Phlebovirus</td>
<td></td>
<td>Rift Valley fever (RVFV)</td>
<td>M</td>
<td>A</td>
<td>H</td>
<td>Several spp</td>
</tr>
<tr>
<td>Bunyaviridae</td>
<td>Orthobunyavirus</td>
<td>California (CAL)</td>
<td>La Cross (LACV)</td>
<td>M</td>
<td>NA</td>
<td>N</td>
<td>Aedes</td>
</tr>
<tr>
<td>Bunyaviridae</td>
<td>Orthobunyavirus</td>
<td>CAL</td>
<td>Jamestown Canyon (JCV)</td>
<td>M</td>
<td>NA</td>
<td>N</td>
<td>Aedes</td>
</tr>
<tr>
<td>Family</td>
<td>Genus</td>
<td>Complex</td>
<td>Virus</td>
<td>Host</td>
<td>Distribution</td>
<td>Disease</td>
<td>Vector</td>
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<tr>
<td>Bunyaviridae</td>
<td>Orthobunyavirus</td>
<td>CAL</td>
<td>California encephalitis (CEV)</td>
<td>M</td>
<td>NA</td>
<td>N</td>
<td>Aedes</td>
</tr>
<tr>
<td>Bunyaviridae</td>
<td>Orthobunyavirus</td>
<td>CAL</td>
<td>Snowshoe hare (SSHV)</td>
<td>M</td>
<td>NA</td>
<td>N</td>
<td>Aedes</td>
</tr>
<tr>
<td>Bunyaviridae</td>
<td>Orthobunyavirus</td>
<td>CAL</td>
<td>Guaroa (GROV)</td>
<td>U</td>
<td>SA</td>
<td>A</td>
<td>Anopheles</td>
</tr>
</tbody>
</table>

Shadowed fields high-light bird hosted viruses.


Vector: Indicates the main identified genus involved in transmission.

Other mosquito-borne viruses with unclear human impact:

- Alpha viruses: Fort Morgan (FMV), Highlands J (HJV), Middelburg virus (MIDV), Ndumu virus (NDUV), Igbo-ora virus, Semiliki Forest (SFV).
- Flavi viruses: Alfuy (ALFV), Bagaza (BAGV), Banzi (BANV), Bussuquara virus (BSQV), Cacipacore virus (CPCV), Edge Hill virus (EHV), Gan Gan (GGV), Ilheus (ILHV), Kokobera (KOKV), Koutango virus (KOUV), Lammi (LAMV), Ntaya (NTAV), Rocio virus (ROC1), Sepik (SEP1), Spondweni (SPO), Stratford virus (STRV), Trubanaman (TRUV), Wesselsbron (WSBL), Yaounde virus (YAOV).
- Orthobunya viruses: Batai (BATV), Bunyamwera (BUNV), Bwamba (BWA1), Cache valley (CVV), Garissa, Germiston (GERV), Ilesha (ILEV), Inkoo (INKV), Itaqui (ITQV), Lednice (LEDV), Madrid (MADV), Marituba (MTBV), Murutucu (MURV), Ngari (NRI), Nepuyo (NEPV), Oriboca (ORIV), Oropouche virus (OROV), Shuni virus (SHUV), Tahyna (TAHV), Wyeomyia (WYOV).

Figure 2. Annual number of clinically and serologically defined infections caused by Sindbis virus in Sweden (1981–2014) and Finland (1974–2014). Sindbis infections are notifiable in Finland, since 1995, whereas in Sweden they are not. Suggested outbreak years are indicated with *.
Ecology of mosquito-borne bird viruses

Mosquito-borne bird viruses infecting humans (and horses) possess both an enzootic transmission in the host population of local birds, and a tangential transmission where bridge-vectors bring the zoonotic virus from birds to humans and other mammals. The enzootic transmission rely on specialized ornitophilic mosquito species, while the tangential transmission rely on opportunistic species feeding on both birds and mammals. When the viral load increases in the bird host population, the probability that there will be transmission to incidental hosts (tangential transmission) also increases. Hence, for transmission to humans to occur, there first need to be an active circulation and amplification of the virus within the bird population (Mullen and Durden 2009).

Many viruses in this group are ecological generalists, capable of infecting a wide array of vertebrate hosts, including several groups of animals besides birds, such as mammals, reptiles and amphibians (Klenk et al. 2004, Bingham et al. 2012, Pérez-Ramírez et al. 2014), and arthropod species (Lawrie et al. 2004, Hayes et al. 2005). However, since further transmission is dependent on many factors both within the hosts and vectors, and in their relation to each other, a limited number of species are important for transmission. It is therefore crucial to discriminate between detection of antibodies or viruses in wild populations and defining the true host or vector (Turell et al. 2005).

Bird hosts

The term vertebrate host can be used with many definitions. It can describe a species found with neutralizing antibodies in nature (potential host), a species that is able to infect a vector (competent host), a species that is maintaining the virus transmission at a low but steady rate (reservoir host), or a species that is increasing the virus transmission (amplification host).

A competent host is defined by its ability to produce a viremia of sufficient magnitude to infect the vector mosquito. For WNV it requires a viremia of approximately $10^5$ PFU/mL to infect Culex species (Goddard et al. 2002), and $10^5$ PFU/mL for SINV to infect Aedes cinereus, or even as low as $10^3$ PFU/mL to infect Cx. torrentium (Lundström et al. 1990a, Turell et al. 1990). Also, important hosts should produce high titer viremias since there is a positive correlation between viremia in the first host and the mosquitoes’ ability to transmit the virus further to a second host (Lundström et al. 1990a, Turell et al. 2001, Goddard et al. 2002). Ideally, the viremia is also of long duration so that as many vectors as possible have the possibility to feed and get infected (Marquardt et al. 2004).

In addition, several ecological factors need to be assessed in the identification of important hosts. The host must be readily fed upon by infective
vectors (Molaei et al. 2006, Gray et al. 2011, Molaei et al. 2013), and it must be prevalent where and when the vector is actively feeding, i.e. forest floor versus canopy, and day versus night (Jupp et al. 2001, Marquardt et al. 2004). Amplifying hosts must be in proximity to humans i.e. within flight range of the bridge vector, for tangential transmission to be a risk (McLean and Scott 1979). Further, it has been proposed that hosts that do not exhibit effective defense behavior towards the vector, such as hatchlings, are more important (Lundström et al. 1993, Caillouët et al. 2013). Chicks are also capable of producing higher viremias due to the small dilution factor in their smaller blood volumes, and having minimal feather coverage that protects them from mosquito bites (Marquardt et al. 2004, Pérez-Ramírez et al. 2014).

Based on these criteria, birds belonging to the order Passeriformes are considered the main reservoir and amplifying hosts of many of the bird-borne alpha- and flaviviruses (Pérez-Ramírez et al. 2014, Molaei et al. 2006, Komar et al. 2004, Reisen et al. 2005). Antibodies to WNV, Saint Louis encephalitis virus and Western equine encephalitis virus has been found in more than 100 species in North America and many of these can also produce viremias high enough to infect mosquitoes (McLean and Scott 1979, Reisen et al. 2000, Komar et al. 2003). Several peridomestic birds such as house sparrows (Passer domesticus) and house finches (Carpodactus mexicanus) are considered important hosts to all of these viruses, since they are often infected in nature, competent, and abundant in proximity to humans (McLean and Scott 1979, Reisen et al. 2000, Hayes et al. 2005). Sometimes flaviviruses have caused massive bird death, such as WNV in corvids in North America (Komar et al. 2003) and Usutu in blackbirds (Turdus merula) in Austria (Chvala et al. 2007), but the species showing symptoms are not necessarily the most important species for spreading the virus. Usually, the reservoir host does not develop disease (Marquardt et al. 2004), and additional species can also be involved in the movement and spread of the virus across the continent (Komar et al. 2003, Rapole and Hubálek 2003).

Bird hosts for Sindbis virus

For SINV in Sweden, antibody prevalence and viremia profiles have been thoroughly investigated. Antibodies to SINV have been found in many species of wild-caught birds belonging to Passeriformes (passerines), Galliformes and Anseriformes, with the highest prevalence (27%) in Passeriformes (Lundström et al. 1992, Francy et al. 1998, Lundström et al. 2001). Antibodies in passerines are only detectable between five days to three months post infection, thus only infections in the same season as sampling can be detected, implying that infection prevalence in passerines, compared to other commonly infected birds, can be underestimated (Lundström and Niklasson 1996). Interestingly, antibodies in birds are detected approximately six weeks before the virus have been isolated from mosquitoes, showing
SINV activity in the bird population already in middle of June (Lundström et al. 2001).

The viremia profiles for species belonging to the same three bird orders were investigated by Lundström et al. (1993). Species within all orders produced viremia titers sufficient to induce high transmission rates in enzootic mosquito vectors. Passerines also had long duration viremias with titers sufficient to induce high infection rates also in the bridge vectors.

Among the passerines, especially thrushes (Turdus), specifically fieldfare (Turdus pilaris), redwing (Turdus iliacus) and songthrush (Turdus philomelos) have been identified as the main amplifying host of SINV in Sweden since they have high and long lasting viremias and the highest antibody prevalence (22–43%). They are also very common in the endemic area and nest in close proximity to humans and vectors (Lundström et al. 1992, Lundström et al. 2001). Also in South Africa, the most commonly infected species is a thrush, i.e. the olive thrush (Turdus olivaceus) (McIntosh 1976).

In Finland, screening of blood samples from game birds have shown high prevalence of antibodies against SINV in black grouse, leading to the hypotheses that this is the main amplifying host in Finland (Brummer-Korvenkontio et al. 2002, Kurkela et al. 2008). Sindbis virus antibodies have been found in grouse also in Sweden (Lundström et al. 1992) and grouse has shown viremia profiles similar to passerines (Lundström et al. 1993, Lundström et al. 1996). However, there are several factors that remain to be investigated before concluding that transmission is dependent on different hosts in the two countries. To date, there is not much data on the SINV prevalence in Finnish passerines to compare with, and lack of evaluation of the ecological constrains of potential hosts, i.e. proximity between hosts, vectors and humans.

Mosquito vectors

Mosquitoes have either of two major vector roles: as enzootic vectors that build up the viral load in the bird host population, or as bridge vectors that occasionally transfer the virus from the bird host to dead-end hosts such as humans or horses. The prerequisite for any further transmission to occur is thus the transmission done by ornithophilic mosquitoes, such as species belonging to the Culex and Culiseta genera (Turell et al. 2005). They are often collected in higher numbers or proportions in traps placed in the tree canopy rather than at 1.5 m height, reflecting their adaptation to their tree-dwelling blood-meal hosts (Service 1971a, Lundström et al. 1996, Šebesta et al. 2010, Johnston et al. 2014).
**Vector identification**

Mosquitoes of many species are commonly found infected with viruses, however a virus isolate alone cannot conclude vector status. This is well exemplified by WNV that has been isolated from 59 different mosquito species in North America, and less than 10 species are considered vectors (Hayes et al. 2005). When collecting mosquitoes for virus screening, preferably all mosquitoes are first identified to species, so that the percentage of infected individuals within a population of a species, i.e. field infection rate (IR), can be calculated.

Species with comparably high infection rates can be important vectors if several additional criteria are fulfilled, referred to as vectorial capacity. Vectorial capacity includes factors such as abundance, longevity, blood-meal host preference and vector competence. Vector abundance is often related to the probability of an infectious bite (Kramer and Ebel, 2003), and the longer a vector lives, the more potential blood meals it can take. A vector species also needs to regularly feed on the reservoir host, as well as the dead-end host for bridge vectors (Turell et al. 2005).

When a mosquito takes a blood meal (Figure 3), the blood is transferred through the proboscis and into the midgut (stomach). The epithelial cells in the midgut wall make up a barrier (the midgut barrier) that the virus first need to infect to also infect the mosquito. If the virus can escape from the basal side of these cells, it can disseminate and spread to other inner organs (Myles et al. 2004, Mahmood et al. 2006, Richards et al. 2012a, Turell et al. 2006). The crucial organ for a vector is the salivary glands and there are studies showing both a barrier for infection of the salivary glands and a barrier for escaping the salivary glands (Myles et al. 2004)

![Figure 3. Steps of infection and infection barriers inside a female mosquito. 1. Ingested infectious blood meal. 2. Infection of the epithelial cells of the midgut wall. 3. Multiplication of virus in the epithelial cells and release. 4. Secondary amplification in other inner tissues. 5. Infection of salivary glands. 6. Multiplication and release from salivary glands into the saliva being secreted when feeding. (Modified from Kramer and Ebel 2003.)](image-url)
In vector competence experiments (Figure 4), mosquitoes are offered a blood-meal containing the pathogen under study, and are kept for varying numbers of days (extrinsic incubation period) under different temperatures, before either being offered a second blood meal from a susceptible host or forced to salivate. The percentage of mosquitoes that can get a disseminated infection (dissemination rate) is estimated through isolating virus from the legs of the mosquitoes. The percentage of mosquitoes that can excrete virus with their saliva (transmission rate) is estimated either through seroconversion or observed infection of the second host, or through virus detection in the mosquito saliva (Goddard et al. 2002, Turell et al. 2006, Balenghien et al. 2008).

A vector competence study also highlights the many things besides the physiological barriers of the mosquito that can influence the transmission rate; such as ingested virus dose (which depends on the amount of virus in the blood of the host), temperature, and duration of the extrinsic incubation period (Goddard et al. 2002, Anderson et al. 2010, Richards et al. 2012b). Generally, the transmission rate increases by feeding on a highly viremic host and staying in high temperature for a long time before feeding on the next host. Vector competence is often found to vary between strains of the same virus (Moudy et al. 2007) as well as between mosquito populations within the same species (Kramer and Ebel 2003, Richards et al. 2012b), thus should preferably be determined for mosquito species and viral strains occurring in a specific geographical region.

**Figure 4.** Three measurements of vector competence.
<table>
<thead>
<tr>
<th>Subgenus</th>
<th>Group</th>
<th>Subgroup</th>
<th>Complex</th>
<th>Name</th>
<th>Distribution</th>
<th>Pathogen involvement</th>
<th>Host</th>
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<td>WNV</td>
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<td>WNV</td>
<td>B</td>
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<td>RVF, WNV</td>
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<td>Cx. vishnui Theobald</td>
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<td>Vishnui</td>
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<td>Tarsalis</td>
<td>Cx. declarator Dyar and Knab</td>
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<td>SLE</td>
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<td>VEE</td>
<td>M</td>
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</tbody>
</table>
Note that not all species belong to a sub-group or a complex.
*earlier known as Cx. fatigans.
Pathogen involvement: see table 1.
Host: B: bird, M: mammal, O: opportunistic (Note that the host preference often is a geographically varyable trait).
Vectors of Sindbis virus

The potential vectors of SINV naturally differ with the different mosquito fauna present in the two endemic areas of human disease; South Africa and Northern Europe. The main vector in South Africa is Cx. univittatus (Jupp 2001). In northern Europe, SINV has been isolated from unidentified mixtures of the ornitophilic species Cx. pipiens/torrentium and Cs. morsitans, and from the opportunist species Ae. cinereus and Ae. rossicus (Jaenson and Niklasson 1986, Francy et al. 1989, Lundström et al. 2014).

Sindbis virus vector competence studies have been performed on Cx. pipiens, Cx. torrentium, Ae. cinereus, Ae. communis and Ae excrucians (Lundström et al. 1990a, Lundström et al. 1990b, Turell et al. 1990). These showed that Cx. torrentium was an outstanding vector with 86-100% infection rate and 100% transmission rate even at low titers in the infectious meal (3 PFU/mL), short duration of extrinsic incubation (14 days) and low temperature (10°C). Cx. pipiens needed higher titers and temperatures to reach a maximum of 57% infection rate and 37% transmission rate. All Ae. excrucians and 50% of the Ae. cinereus mosquitoes developed a disseminated infection but only 50% of these transmitted the virus. Aedes communis had a 75% dissemination rate but did not refeed in the experiment (Turell et al. 1990).

Thus, with the current knowledge from field infection rates, vector competence experiments and mosquito ecology, the potential enzootic vectors of SINV are Cx. torrentium, Cx. pipiens and Cs. morsitans (however lacking in vector competence estimation), and the potential bridge vectors are Ae. cinereus and Ae. rossicus (also lacking in vector competence estimation). Despite a number of studies on the mosquito vectors, the knowledge has been hampered by confusion regarding the Culex species involved in the enzootic transmission. Only the vector competence experiments have been able to secure information on species level, while species-specific field infection rates, occurrence and prevalence data are lacking due to difficulties in morphological identification of the two Culex species. Females of Cx. pipiens and Cx. torrentium are only separated by a few prealar scales, which most freshly hatched Cx. torrentium have, and Cx. pipiens lack. However, these scales are easily rubbed off and thus, most mosquitoes with time look like Cx. pipiens (Service 1968). Mosquito inventories in Sweden have therefore only information on unidentified mixtures of Cx. pipiens/torrentium (Lundström et al. 2013).

Culex mosquitoes as vectors

All of the mosquito-born bird viruses have Culex (Culex) species as their mosquito vector, except two (Eastern equine encephalitis virus and Highlands J virus), which both utilize Cs. (Climacura) melanura (Lundström and
The Culex genus includes 768 species divided into 26 subgenera. More than 28 species have been reported in pathogen transmission (Table 2), which includes viruses and filarial nematodes, e.g. Wuchereria bancrofti (Harbach 2011). Five species (Cx. theileri, Cx. univittatus, Cx. pipiens, Cx. modestus, Cx. torrentium) and one biotype (Cx. pipiens molestus) of potential vectors from the Culex genus can be found in Europe.

Morphologically similar species
The systematics within the Culex genera is complicated and constantly changing, with several groups containing species that are so similar that it is impossible to reliably identify field collected material morphologically (Harbach 2011). Often, females are indistinguishable while males can be separated based on characters of their genitalia. This is however of limited use since most sampling techniques are directed to catching females, and some species also hybridize in zones where they overlap. Therefore there is a lack of consensus regarding concepts of species, subspecies and biotypes (Harbach 2011).

Mosquitoes in the Cx. pipiens complex are the most widely distributed mosquito species in the world. Culex pipiens, the northern house mosquito, occurs in all temperate regions of the world and at higher altitude regions in Africa, while Cx. quinquefasciatus (old synonym: Cx. fatigans), the southern house mosquito, is found in warm temperate to tropical regions all over the world (Harbach 2012). Females of the two species are not distinguishable, and also hybridize in some areas where they co-occur (i.e. in North America and in Asia). In Asia, the hybridization took place a long time ago and the hybrids often go under the name Cx. pallens, which by some authors is regarded as a separate species (Harbach 2012, Turell 2012).

In northern and central Europe, there is one other species (Cx. torrentium) and one biotype (Cx. pipiens molestus) that can be morphologically confused with Cx. pipiens. Most authors identify the biotype Cx. pipiens molestus by five criteria separating it from Cx. pipiens: it is breeding underground (hypogeous), it is able to mate in confined spaces (stenogamous), it is able to lay eggs without taking a blood meal (autogenous), it overwinters without diapause (homodynamic), and it readily bites humans (mammalophilic) (Byrne and Nichols 1999, Fonesca et al. 2004). Based on these criteria, populations of Cx. pipiens found in underground environments, such as the London railway tunnels or cellars, have been identified as Cx. pipiens molestus (Byrne and Nichols 1999). However, the definition and identification of Cx. pipiens molestus is in a confused stage, with some authors considering it as a separate species, and many studies neglecting to refer to the criteria that have been used for species identification. Since the same neglect surrounds the morphologically identical Cx. torrentium, the knowledge of the distribution and vector roles of these two species and one biotype in Europe is in a very unreliable stage.
In the United States there are several morphologically very similar species, including *Cx. pipiens*, *Cx. quinquefasciatus*, *Cx. restuans*, *Cx. salinarius* and *Cx. nigripalpus*, that besides the biotype *Cx. pipiens molestus*, play important roles in the transmission of different viruses in different parts of the country (Apperson et al. 2002, Rochlin et al. 2007, Farajollahi et al. 2011). *Cx. restuans* occurs in eastern and central United States, and in these areas, the identification problem is similar to that of *Cx. torrentium* in Europe, with relatively little species-specific knowledge on *Cx. restuans*, which is routinely assumed to be similar to *Cx. pipiens* (Harrington and Poulson 2008). Both *Cx. pipiens* and *Cx. restuans* are involved in WNV transmission and arbovirus surveillance programs often allow combined poole of *Cx. pipiens* and *Cx. restuans* to be tested for WNV (Rochlin et al. 2007).

Many species within the *Cx. pipiens* group have historically been considered mainly ornithophilic, but recent studies have shown unexpected variation in feeding patterns of populations from different geographical origin, likely dependent on both genetic factors and the availability of hosts (Farajollahi et al. 2011). In Europe, *Cx. pipiens/torrentium* (as yet, there are no studies on *Cx. pipiens/torrentium* identified to species) take their blood meal exclusively from birds (Service 1971b, Jaenson and Niklasson 1986), similar to *Cx. pipiens* in North America (Apperson et al. 2002, Molaei et al. 2006, Farajollahi et al. 2011.). *Cx. quinquefasciatus* appears to be more versatile in the choice of host, feeding on both birds and mammals in parts of North America and South Africa (Donaldson 1979, Farajollahi et al. 2011). Thus, depending on the geographical area, *Culex* species can also play a role as bridge vector, in addition to their historically known importance as enzootic vectors. Some species also display a change in host preference over the season. (Kent et al. 2007).
Aims

The main aim of this thesis was to clear the confusion that has surrounded the vector species *Cx. pipiens* and *Cx. torrentium*. Prior to these works, *Culex* catches in Sweden have been labeled “*Cx. pipiens/torrentium*” to indicate the unknown potential mixture of species. Likewise, many reports from Europe have used the same expression, or labeled the catch *Cx. pipiens* without presenting any specific identification effort although that both species are part of the country fauna. Since the two species show great differences in vector competence for SINV, it was crucial to establish a new platform for discussions on mosquito-born bird viruses in Europe.

The first aim was to develop a molecular method of species identification that reliably could separate the two species, based on identified males as reference material. After that, this new method was used for a number of purposes:

- For a thorough investigation of *Cx. pipiens/torrentium* in Sweden and Finland, the endemic countries of SINV, determining the geographical distribution and relative abundance of the two species.
- For screening of *Cx. pipiens/torrentium* in 11 other European countries and extrapolation of the European distribution of the two species.
- For investigating the potential biases introduced when using different trapping methods to collect and monitor the abundance of the two species.
- For the first time isolating SINV from species-identified *Culex* mosquitoes in Europe and thereby contributing with crucial information on their vector status.
Materials and Methods

Collection of samples
Mosquitoes were collected from the field using three types of traps (Figure 5). Adult females were collected using the Centers for Disease Control and Prevention (CDC) light trap baited with carbon dioxide (CDC-traps) and Gravid traps baited with hay infusion. The former attracts host-seeking females of many different mosquito species, while the latter is designed to attract gravid *Culex* females ready to lay their eggs. Larvae were collected by Oviposition traps (Ovitraps), designed to attract *Culex* females to lay eggs that hatch to larvae, consisting of black five liter plastic buckets baited with hay infusion, or directly from the field from a number of different habitats such as car tires, tractor scoops, water buckets etc. Adult *Culex* males were collected with insect nets.

Collections of females with CDC-traps were mainly performed within the regular mosquito surveillance program in the River Dalälven floodplains area (Lundström et al. 2013), with addition of a similar surveillance program in Kristianstad. Gravid trap and Ovitrap collections were performed in both River Dalälven floodplains and in Kristianstad. Field collections of larvae were made in Sweden, Finland, Denmark, Germany, France, Switzerland, Croatia, England, Holland, Belgium, Serbia, and Poland and Czech Republic. *Culex* males were only collected in Czech Republic.

Species identification
Males were identified to species based on diagnostic characters of the male genitalia (Service, 1968). Eighteen of these males (ten *Cx. torrentium* and eight *Cx. pipiens*) served as template material for the development of a restriction enzyme assay for molecular species identification of females and larvae of the two species.

Adult females and larvae were first morphologically identified to *Cx. pipiens/torrentium* on a chill table using a stereo-microscope and keys by Becker et al. (2003), and sorted out from the rest of the mosquito catch and stored in −80°C. Individual whole mosquitoes, or only legs, were then used for DNA extraction using the E-Z 96® Tissue DNA Kit (Omega Bio-Tek, Inc., Norcross, GA, USA) following bead beating. The COI-3′ region (795
base pairs) of mitochondrial DNA of all samples was amplified by PCR using the primer pair C1-J-2183 and TL2-N-3014 (Simon et al. 1994).

For development of the species identification assay, the PCR products of 18 males and 44 females were also sequenced on a Megabace 1000 automated sequencer using a DYEnamicTM ET Dye Terminator Kit (MegaBACETM) (GE Healthcare UK Ltd, Chalfont, UK).

The PCR products were then divided into two tubes and incubated with two different restriction enzymes, FspBI (BfaI) and SspI (Fermentas International, Inc.), each making one cut in the product of either species and leaving the other species uncut (Figure 6). The resulting fragments were run by gel electrophoresis, either manually on a 1.5% agarose gel and visualized with ethidium bromide, or on a QIAxcel analyser using the QIAxcel DNA Screening Kit (Qiagen, Inc., Valencia, CA, USA).

Screening for viruses

All females of *Cx. pipiens*, *Cx. torrentium* and *Cs. morsitans* collected between July 13 and September 13, 2009 (n=958), were screened for SINV. Individual specimens of *Cx. pipiens/torrentium* were processed by removal of the legs, which were used for DNA extraction and identification to species

**Figure 5.** Three mosquito trap types used in this thesis: CDC Light trap baited with carbon dioxide, Gravid trap baited with hay infusion, Oviposition trap also baited with hay infusion.
Figure 6. The species-diagnostic banding pattern on an agarose gel for *Culex pipiens* and *Cx. torrentium* after restriction enzyme digestion of the mitochondrial COI gene. Lane 1 (*Cx. torrentium*) and lane 2 (*Cx. pipiens*) after digestion with enzyme FspBI. Lane 4 (*Cx. pipiens*) and lane 5 (*Cx. torrentium*) after digestion with enzyme SspI. Lane 3 is a 100-bp ladder for reference.

while the bodies were used for RNA extraction from individual specimens.

Single or pooled mosquitoes (301 *Cx. torrentium*, 367 *Cx. pipiens* and 74 pools of *Cs. morsitans*) were mixed with 500 µl of phosphate-buffered saline (PBS), 20% fetal calf serum, 250 µg/ml Amphoterecin B, 100 U/ml penicillin and 100 µg/ml streptomycin, and homogenized by bead beating. After centrifugation, 50 µl of the supernatant was taken and mixed with 350 µl of RLT buffer from RNeasy Mini kit (Qiagen). The remaining supernatant was stored at −80°C until virus isolation. RNA was extracted from the RLT mixture using RNeasy Mini kit for the *Culex* mosquitoes, and MagAttract RNA Tissue Mini M48 kit on a BioRobot M48 workstation (Qiagen) for the *Culiseta* mosquitoes.

All samples were screened for SINV using a q-RT-PCR with primers and probe described by Jöst et al. (2010). First, all samples were screened by pooling two to ten RNA extractions by species and week. Second, a subsequent q-RT-PCR was run with all individual samples from the pools that were found positive by the first screening. Thereby individual mosquitoes (*Culex*) or pooled mosquitoes (*Culiseta*) were ultimately tested for SINV-RNA.

All samples that were positive in the q-RT-PCR were inoculated onto African green monkey kidney (Vero) cells for virus isolation attempts. The
cells were incubated at 37°C for one hour before addition of 10 ml of Minimum Essential Media with Earl’s salts supplemented with 1% HEPES, 1% Penicillin-Streptomycin mixture and 5% fetal calf serum (Life Technologies). The cells were further incubated at 37°C and observed for cytopathic effect (CPE) twice daily. The medium from cultures showing CPE was removed, centrifuged and stored at −80°C until sequence analysis.

Part of the SINV Envelope 2 glycoprotein gene was amplified using a one-tube RT-PCR and the primers from Norder et al. (1996). The resulting cDNA-products were sent to Macrogen Europe (Amsterdam, the Netherlands) for sequencing.

Phylogenetic and statistical analyses

For the Culex species identification assay, the sequences were edited with Codon Code aligner (Codon Code Corp., Dedham, MA, U.S.A.) and the alignment and the evaluation of theoretical restriction sites were performed with BioEdit Sequence Alignment Editor (Hall 1999). To ascertain that the different species grouped clearly together in a phylogenetic tree with strong support, a 795-bp sequence of the mitochondrial COI gene was used in a phylogenetic analysis of 67 Culex sequences representing two species together with four sequences from outgroup species (two Aedes species and two Culiseta species). The sequences included were either sequenced by the author or downloaded from GenBank. Bayesian phylogenetic analyses were performed with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001).

For all mosquito sampling sites, longitude and latitude were identified by using a hand-held GPS, or from Google Earth (2011). Additional information on altitude, temperature (mean, minimum and maximum), and cumulative precipitation was extracted from WorldClim (Hijmans et al. 2005) and analyzed in ArcGIS 9.2 (Environmental Systems Research Institute, 2007).

All statistical analyses were performed in SAS 9.2 (SAS Institute, Inc., 2008). Analyses included the geographical variations in the detection (0=not detected, 1=detected) of larvae or adults of each of the two species and their abundances relative to one another, using the proportion of one species at a sample site as the dependent variable. The distributions of the species were also analyzed in relation to altitude, habitat type (natural or artificial), day and year of sampling, cumulative precipitation, mean, minimum and maximum temperatures, and the length of the growing season (Rötzer and Chmielewski 2001).

To compare the composition of species caught in different trap types, all catches from each trap type and region were summarized for each sampling occasion. The proportion of Cx. torrentium was used as dependent variable, in a generalized linear model with binomial distribution, with trap type and sampling week as explanatory factors.
The occurrence of SINV in field-collected mosquitoes was evaluated as infection rates (IR=[number of positive individuals/number of mosquitoes tested]*1000) for Cx. torrentium and Cx. pipiens, and as Minimum infection rates for Cs. moritans (MIR=[number of positive pools/total number of mosquitoes tested]*1000).

The phylogenetic analysis of the SINV isolates included all the sequences isolated by the author together with 63 additional virus strains downloaded from GenBank and previously published by Lundström and Pfeffer (2010). Bayesian phylogenetic analyses and maximum likelihood analyses were performed with MrBayes 3.2.1 and Garli 2.0.
Results and discussion

A restriction enzyme assay to distinguish between the mosquitoes *Cx. torrentium* and *Cx. pipiens* (Paper I)

In the first paper we developed a reliable method for identifying *Cx. pipiens* and *Cx. torrentium* to species. This study aimed for transparency in reference material and definitions of species, using morphologically identified males as template material for establishment of the assay. The restriction enzyme FspBI was used for cutting specific PCR products of *Cx. torrentium*, while *Cx. pipiens* remains uncut. Identification of *Cx. pipiens* was confirmed by digestion of the restriction enzyme SspI, which leaves *Cx. torrentium* undigested (Figure 6). The assay was evaluated on 227 Swedish samples that all resulted in two separate and consistent groups. The sequencing of 44 arbitrarily chosen specimens verified that the two groups were correctly divided with high support into *Cx. torrentium* or *Cx. pipiens*.

The quality of the results from the restriction enzyme assay was independent of the amount of starting material; hence small amounts such as only the legs of a mosquito was sufficient for species identification. The assay was an essential tool for further studies on these species and was used in all the other subprojects within this thesis.

Geographic distribution and relative abundance of *Cx. torrentium* and *Cx. pipiens* in Sweden (Paper II)

In the second paper, Swedish *Cx. pipiens/torrentium* was thoroughly investigated, using both larvae collected from 49 field sampling sites distributed from Torneå in the north to Kristianstad in the south (Figure 7) and adult females collected with CDC-traps. In total, 1012 larvae were identified to species and overall, *Cx. torrentium* dominated (89%) over *Cx. pipiens* (11%) (p<0.001), and occurred in all but one sampling site. The proportion of *Cx. torrentium* was the highest in the north (99%) and decreased with decreasing latitude (p<0.001) and altitude (p=0.004) and was at the lowest
83%, in the far south of Sweden. The proportional latitudinal decrease of *Cx. torrentium* was due to the increase in presence of *Cx. pipiens* further south, i.e. *Cx. pipiens* was found together with *Cx. torrentium* at more sites in the south. Thus, there was no decrease in the presence of *Cx. torrentium*.

Previously, both *Cx. pipiens* and *Cx. torrentium* males have been found at an altitude of 1500 m in the Pyrenées (Sicart 1954), Thus, high altitude per se is not a limiting factor. Rather, the limited distribution of *Cx. pipiens* in Sweden is likely to be caused by underlying factors connecting altitude and latitude, such as the temperature. We found that air temperature was negatively correlated with both altitude and latitude and thus, positively correlated with the presence of *Cx. pipiens*.

*Cx. torrentium* is known as a species adapted to cold habitats, based on a few empirical studies, (von Struppe 1989), and to occur at high altitude (Sicart 1954). However, a more recent study found that *Cx. torrentium* larvae occurred in Germany at a mean water temperature of 24°C, ranging be-
tween 21.3°C and 33.7°C (Küpper et al. 2006). These results showed that *Cx. torrentium*, besides being cold tolerant, also can persist under warmer conditions. The upper limit of *Cx. torrentium*’s temperature range, and its southern geographical limits, are not known, but the species has been found in Southern Europe, Iran, and Iraq (Harbach 1988). Within Sweden, we did not observe any southward decrease in the presence of *Cx. torrentium*.

Both species were commonly found together in artificial containers such as garden water barrels, garden ponds, car tires, manure water pools, concrete tubes, tractor scoops and water tubs for animals. In contrast, published data on *Cx. torrentium* is very scarce and much of the information available is anecdotal. A common assumption has been that it is a clean water species, even though the literature include several examples where larvae have been found in a variety of aquatic habitats, including ponds rich in organic content and sometimes together with *Cx. pipiens* (Scherpner 1960, Gillies and Gubbins 1982, Ishii and Sohn 1987, von Struppe 1989, Raymond 1995).

This study also included 199 adult females that were collected in southern and central Sweden and identified to species. Overall, *Cx. pipiens* dominated the adult catches (79%), but there were big differences between different regions. As for the larval data, the presence of *Cx. pipiens* decreased with latitude (p=0.02) from 93% in Kristianstad in the south to 50% in the Nedre Dalälven region. The striking difference in species-composition between the larval and adult samples was unexpected, and gave inspiration for a further study on sampling methods (paper IV).

This study also showed that the overall number of *Cx. pipiens/torrentium* females (not identified to species) caught in CDC-traps decreases with latitude (p=0.001) with on average 44 females caught per trap and night (trap-night) in the south and 0.24 females/trap-night in the north. The overall country mean is 4.0 females/trap-night, and only at one rare occasion in Kristianstad the number reached as high as 1434 females/trap-night. Species identification of a subset of this catch (n=60) showed that 92% were *Cx. pipiens*.

**Table 3.** The percentage of all specimens identified as *Culex torrentium* and *Cx. pipiens*, as larvae and adults, summarized for four different vegetation zones in Sweden (A: Nemoral, B: Boreo-nemoral, C: Southern boreal, D: Middle boreal. See Figure 7).

<table>
<thead>
<tr>
<th>Species</th>
<th>Stage</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cx. torrentium</em></td>
<td>larvae</td>
<td>92</td>
<td>83</td>
<td>90</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>adults</td>
<td>7</td>
<td>27</td>
<td>50</td>
<td>n/a</td>
</tr>
<tr>
<td><em>Cx. pipiens</em></td>
<td>larvae</td>
<td>8</td>
<td>17</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>adults</td>
<td>93</td>
<td>73</td>
<td>50</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Absence of sampling is indicated by n/a.
**Culex torrentium** is more prevalent than *Cx. pipiens* in northern and central Europe (Paper III)

In paper III, a large-scale sampling of *Cx. pipiens/torrentium* larvae was performed at 138 sampling sites in 13 European countries by the author and several collaborators in their home countries. The samplings resulted in 2559 larvae of *Cx. pipiens/torrentium* that were identified to species using our molecular method (paper I; Figure 8 and 9). The earlier described dominance of *Cx. torrentium* in Sweden (paper II) was similar in Finland, and the probability of detecting larvae of *Cx. torrentium*, and the proportion of *Cx. torrentium* within the samples, increased with latitude, while the probability of detecting larvae of *Cx. pipiens* decreased with latitude. Hence, the indicated distribution pattern from paper II was extended further south in Europe with *Cx. torrentium* dominating in northern Europe and *Cx. pipiens* dominating in the south. Both species occurred together in major parts of Europe (45 sites), with a transition in species dominance in central Europe, where both species are roughly equally common, which is in agreement with the findings of a recent study by Weitzel et al. (2011). *Culex torrentium* was lacking in our samples from areas south of the Alps mountain range. But, since there are previous records of *Cx. torrentium* from countries around the Mediterranean, including Turkey, Greece, Italy, Portugal and Spain (Parrish 1959, Samanidou-Voyadjoglou and Darsie 1993, Snow and Ramsdale 1999, Aranda et al. 2000), we conclude that *Cx. torrentium* is present, but rare, in the Mediterranean area.

The best predictor of the overall geographical distribution pattern of both species in Europe was the length of the growing season, i.e. when the majority of bushes and trees are proliferating. This is calculated by an equation based on longitude, latitude and altitude (Rötzer and Chmielewski 2001), thus, it theoretically combines factors that we in both paper II and III have seen important for the presence of these species. The detection and proportion of *Cx. torrentium* larvae decreased with the length of the growing season, while the probability of detecting *Cx. pipiens* larvae in a sample increased with the length of the growing season. Based on the length of the growing season, we extrapolated the occurrence of *Cx. pipiens* and *Cx. torrentium* in the whole of Europe. The length of the growing season ranged between 152 days and 212 days at sites where *Cx. torrentium* was found, and between 163 days and 231 days at sites where *Cx. pipiens* was found. These values were used as class limits to visualize the expected areas with suitable length of growing season for the respective *Culex* species (Figure 10).
Figure 8. Distribution of *Culex torrentium* in Europe.

Figure 9. Distribution of *Culex pipiens* in Europe.
Figure 10. Extrapolation of the potential distribution of *Culex torrentium* and *Cx. pipiens* in Europe based on the findings of our study and on the identified significant correlation with the length of the growing season at the sample sites. The map shows areas that could potentially host both species, areas where only either *Cx. torrentium* or *Cx. pipiens* would occur, and areas that have a growing season not represented in this study and thus for which extrapolation was not possible.

The increased presence of *Cx. torrentium* in areas with shorter length of the growing season might also be the explanation for the decrease in detection and proportion of *Cx. torrentium* larvae with sampling day. *Culex torrentium* tends to be found more often early in the season and *Cx. pipiens* later in the season, a conclusion also indicated by Gillies and Gubbins (1982) and von Struppe (1989). One potential explanation for the distribution patterns described may be that *Cx. torrentium* has a fixed number of generations per year, whereas *Cx. pipiens* has greater potential to vary its numbers of generations.

The larval habitats investigated were mainly typical of the nutrient-rich, small waterbody environments in which larvae of *Culex* species usually occur. As in paper II, and some previous reports (Gillies and Gubbins 1982, Ishii and Sohn 1987, von Struppe, 1989), *Cx. torrentium* larvae were found significantly more often in artificial water bodies than in natural ones. This stresses the importance not to rely on the type of larval habitat when tentatively determining the species. The sampling sites usually contained only *Cx. pipiens/torrentium* larvae, but other *Culex* species, as well as species of *Ae*
des and Culiseta, shared the larval habitat with *Cx. pipiens/torrentium* at eight sampling sites.

In this study, we also did morphological identification of 1712 adult males collected in the Czech Republic. The results supported those obtained from larval collections; *Cx. torrentium* dominated the samples (67%) overall, and the detection and proportion of *Cx. torrentium* decreased as the season progressed, along with increases in the detection of *Cx. pipiens*. Both species were found in natural and artificial habitats, but the proportions of *Cx. torrentium* were higher in artificial than in natural habitats.

**Trapping biases of *Cx. torrentium* and *Cx. pipiens* (Paper IV)**

In paper IV we evaluated three different trapping procedures (the CDC-trap, the Gravid trap and the Ovitrap) to investigate previous indications (paper I and II) that adult sampling using Light traps catch *Cx. pipiens* and *Cx. torrentium* in different proportions as compared to what is observed in larval sampling.

The comparison showed that the species composition in CDC-traps was not reflected in neither the catch from Gravid traps nor Ovitraps. CDC-traps caught a lower proportion of *Cx. torrentium* than did the Gravid traps, in both Kristianstad (p=0.005) and the River Dalälven floodplains (p=0.01). In the River Dalälven floodplains, CDC-traps also caught a lower proportion of *Cx. torrentium* than what Ovitraps did (p=0.006), while there was no difference between Ovitraps and Gravid traps (p=0.6). Thus, whereas the proportions of the two species are comparable between Gravid traps and Ovitraps, CDC-traps deviate. This is further supported by the comparable species compositions found when identifying adult males and field-sampled larvae (paper III). These results confirmed earlier observations on differences between larval sampling in the field and catches from CDC-traps (paper I and II, Weitzel et al. 2011, Beck et al. 2003). All other sampling methods used described in this thesis provided a higher proportion of *Cx. torrentium* than what CDC-traps do in the same area, suggesting a bias against *Cx. torrentium* in CDC-traps.

In Sweden, larvae of *Cx. pipiens/torrentium* are very easy to find in the field and highly abundant throughout the country. Paper II showed that on average 89% of the larvae are *Cx. torrentium*, and that the chance of finding *Cx. pipiens* larvae and adults increases further south in the country. The number of *Cx. pipiens/torrentium* individuals caught in CDC-traps is very low, and also increases southwards in the country and in Europe (Sudarić Bogojević et al. 2009, Šebesta et al. 2012). This paper gave new perspectives to the variances in size in CDC-trap catches observed in both Sweden and
other European countries. The reason why *Cx. p. torrentium* mosquitoes are collected in higher numbers in CDC-traps in the south than further north could simply be because, in the south, the species that get collected, i.e. *Cx. p. pipiens*, is more common. Further north, fewer individuals are caught as *Cx. p. pipiens* decreases, and the traps miss out on collecting *Cx. torrentium*, even though it is potentially abundant. That would also explain why the CDC-traps in Kristianstad often collect more *Culex* individuals than in the River Dalälven floodplains (Lundström, unpublished) which was also apparent in this study.

### Exceptional Sindbis virus infection rate in Swedish *Cx. torrentium* defines its role as a major enzootic vector (Paper V)

In paper V we present information on the infection prevalence of SINV in the enzootic vectors, *Cx. torrentium* and *Cx. p. pipiens*, for the first time reliably identified to species. This was crucial information lacking in defining their vector status, and an important addition to previous studies of SINV ecology.

The species-specific IR was 36.5/1,000 for *Cx. torrentium* and 8.2/1,000 for *Cx. p. pipiens*, while 21/1,000 for the resulting *Cx. p. pipiens/torrentium* mixed species. The calculated MIR for *Cs. morsitans* was 6.9/1,000 mosquitoes. The significantly higher SINV infection rate in field caught *Cx. torrentium* is an important addition to the previous findings of the extreme susceptibility of *Cx. torrentium* to SINV in the lab, where all infected *Cx. torrentium* were able to transmit the virus to a susceptible host (Lundström et al. 1990a). Thus, the observed natural infection rate of 36.5/1,000 *Cx. torrentium* translates to 36 mosquitoes per 1,000 being able to transmit SINV (Table 4). In contrast, the observed natural infection rate of 8.2/1,000 *Cx. p. pipiens* translates to only two mosquitoes per 1000 being able to transmit the virus, since only one third of the infected *Cx. p. pipiens* (28%, n=212) are able to transmit SINV upon re-feeding (Lundström et al. 1990a).

The observed infection rates of 36.5/1,000 in *Cx. torrentium* and 8.2/1,000 in *Cx. p. pipiens* are very high for both species, with the infection rate in *Cx. torrentium* being one of the highest ever reported in mosquitoes (Jupp 2001). In South Africa, SINV was more prevalent in *Cx. univittatus* (57 isolates, n=30,220) than in *Cx. p. pipiens* (one isolate, n=22,443) and the annual infection rates in *Cx. univittatus* varied between 0.3 to 8.9/1,000 mosquitoes in 1966 to 1976 (McIntosh et al. 1978). In Israel, SINV was more prevalent during 1982–1984 in *Cx. perexiguus* (eight isolates, n=4,824) than in *Cx. p. pipiens* (nine isolates, n=67,621) with an infection rate of 1.7/1,000 in *Cx. perexiguus* (Samina et al. 1986). In Saudi Arabia, SINV was
more prevalent in *Cx. univittatus* (13 isolates, n=2,743) than in *Cx. pipiens* (one isolate, n=7,560), and the infection rate in *Cx. univittatus* was 4.7/1,000 mosquitoes in 1980 (Wills et al. 1985). It is thus quite clear that *Cx. pipiens* is generally a secondary enzootic vector of SINV.

The high infection rates observed in this study could be partly attributed to the fact that single mosquitoes were analyzed, as compared to the more common technique of pooling; hence the discrepancy between infection rate per se and minimum infection rate. The first q-RT-PCR screening revealed 14 pools positive for SINV-RNA. Nine pools contained *Cx. torrentium*, three contained *Cx. pipiens*, and two contained *Cs. morsitans*. The subsequent individual q-RT-PCR revealed 16 SINV-RNA positives, as one of the positive *Cx. torrentium pools*, containing samples from ten individuals, contained three individual SINV-RNA positive mosquitoes. In summary, 11 of the 301 individual *Cx. torrentium*, and three of the 367 individual *Cx. pipiens* were positive for SINV-RNA. For *Cs. morsitans*, two of the 74 pools (containing three and four individuals respectively), were positive for SINV-RNA. Thus, two SINV-RNA positives would have remained undetected if only pooled mosquitoes were analyzed, despite that our original pools were made up of ten individuals only (compared to the more common 50 individuals per pool). The risk of underestimation of the infection rate increases with high virus prevalence in natural vectors, and thereby differences in infection rates between potential vector species could often in fact be greater than what is detectable in pooled samples (Bustamante and Lord 2010).

The remaining supernatants from the 16 SINV-RNA positive mosquitoes were inoculated onto Vero cells, which all showed virus-specific CPE two to four days after inoculation. Thus, SINV strains were successfully isolated from all 16 RT-qPCR positive samples. The phylogenetic analysis showed that all 16 new SINV isolates belonged to the SINV-1 genotype with close relationships to previously isolated strains from Europe, the Middle East and South Africa. No differences in relationships between isolates from different mosquito species were observed.

**Table 4.** Combinations of parameters from vector competence experiments (Lundström et al. 1990a) and studies on natural infection for potential mosquito vectors of Sindbis virus.

<table>
<thead>
<tr>
<th>Species</th>
<th>Artificial infection rate</th>
<th>Artificial transmission rate</th>
<th>Natural infection rate</th>
<th>Potential infectious bites</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cx. torrentium</em></td>
<td>90-100%</td>
<td>100%</td>
<td>36.5</td>
<td>36/1,000</td>
</tr>
<tr>
<td><em>Cx. pipiens</em></td>
<td>4-55%</td>
<td>14-37%</td>
<td>8.2</td>
<td>2/1,000</td>
</tr>
<tr>
<td><em>Cs. morsitans</em></td>
<td>n/a</td>
<td>n/a</td>
<td>6.9</td>
<td>n/a</td>
</tr>
<tr>
<td><em>Ae. cinereus</em></td>
<td>50%</td>
<td>50%</td>
<td>0.7</td>
<td>&lt;1/1,000</td>
</tr>
<tr>
<td><em>Ae. rossicus</em></td>
<td>n/a</td>
<td>n/a</td>
<td>0.4</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Species not tested are indicated by n/a.
Conclusions

This investigation of two *Culex* species in Sweden and Europe has showed that *Cx. torrentium* is a far more common and geographically dispersed species than previously believed. The two *Culex* species often occur together, but in different proportion in relation to latitude. In Scandinavia, *Cx. torrentium* accounts for 89% of the collections, whereas in central Europe both species are equally common. *Culex torrentium* is scarcely found south of the Alps mountain range where *Cx. pipiens* dominates heavily. The uncovered distribution of *Cx. torrentium* is geographically matching the area with SINV occurrence in northern and central Europe, and the area with about 90% *Cx. torrentium* in Sweden and Finland is the endemic area with recurrent outbreaks of SINV infections in humans. Sindbis virus circulates also in other parts of Europe, e.g. Germany where virus has been isolated from mosquitoes, but no cases of human disease have been reported. Thus, the results from this thesis indicate that the endemic areas in Sweden and Finland are ecological hotspots for SINV providing sufficient populations of the ornithophilic enzootic vector *Cx. torrentium*, and of the passerine amplification hosts. The abundance of human cases shows that there are also sufficient populations of the *Aedes* bridge-vectors feeding on both birds and humans in these areas. The threshold abundances of enzootic vectors, bridge-vectors, and amplification hosts, are not reached in central Europe where *Cx. torrentium* is still abundant, but not as dominant.

For the first time, SINV was isolated from individual mosquitoes caught in the field and identified to species by a molecular method. The infection rate for SINV in *Cx. torrentium* was 36/1,000, which is one of the highest infection rates ever reported. This establishes *Cx. torrentium* as the main vector of SINV, since it is now showed to be common, very often infected in nature, and an excellent transmitter. These findings raise the concern if European *Cx. torrentium* populations also can carry other bird-associated viruses. So far, no other virus isolation attempt has been made for species-identified *Cx. torrentium*. However, vector competence studies on *Cx. torrentium* also for the emerging WNV would be highly interesting, especially after the discovery of the unexpected high abundance of *Cx. torrentium* in both north and central Europe reported in this thesis. If *Cx. torrentium* would be a more efficient vector for WNV than *Cx. pipiens* (which is only a moderate vector), the risk of WNV in central and northern Europe is actually higher than estimated at present. If *Cx. torrentium* would turn out to be a less
efficient vector, the risk would be lower than current estimations in central Europe and in southern Sweden, and almost negligible in central and northern Sweden, as well as in Finland.

This thesis has increased the knowledge of the respective vector status of the two species *Cx. torrentium* and *Cx. pipiens*, with information on their distribution, abundance, field infection rates and trapping bias. Also the molecular method for species identification has proved to be a most valuable tool, and will be useful for further studies of these two mosquito species, including species-specific host preferences and abundances in relation to disease outbreaks in humans. Future studies on SINV, as well as on other mosquito-born bird viruses, will also gain from the baseline information provided here, and from the principles of vector discrimination that have been discussed.
Svensk sammanfattning

Skickmyggor är bärare (vektorer) av ett hundratal olika virus som kan infektera människor och djur. Den här avhandlingen handlar om två myggarter ur släktet *Culex*, *Cx. torrentium* och *Cx. pipiens*, som i varierande grad är vektorer för Sindbisvirus. Hos människor kan Sindbisvirus ge utslag, feber och kronisk ledvärk som i Sverige går under namnet Ockelbosjuka. Samma sjukdom förekommer även i Finland (Pogosta), västra delen av Ryssland (Karelsk feber) och Sydafrika (Sindbisfeber). I Sverige diagnosticeras vanligt endast några få upp till några tiotal fall per år, medan hundratals fall har diagnosticerats vissa år i Finland.

I naturen infekteras fåglar av Sindbisvirus via fågelbitande stickmyggor ur släktet *Culex* (enzootisk vektor). När infektionen är tillräckligt spridd i fågelpopulationen ökar risken för att en annan typ av mygga ur släktet *Aedes*, som tar blod från både fåglar och människor, ska infekteras av Sindbisviruset och föra över det till en människa.

Sindbisvirus har flera likheter med andra myggburna fågelvirus. De allra flesta har fåglar ur ordningen tätvingar som värddjur och fågelbitande stickmyggor av släktet *Culex* som enzootiska vektorer. Arter inom släktet *Culex* är ofta förvillande lika, vilket har försvårat möjligheten att urskilja potentiella vektorarter inom släktet. Detta kan dock vara av stor vikt eftersom arterna, trots deras snarlika utseende, kan vara olika bra på att sprida virus. Så är även fallet med Sindbisvirus och vektorerna *Cx. torrentium* och *Cx. pipiens*. Experiment med myggor i labb har visat att *Cx. torrentium* är en avsevärt bättre vektor än *Cx. pipiens*. Nästan alla *Cx. torrentium* infekteras med Sindbisvirus när de stuckit en infekterad fågel och nästan alla sprider det vidare. För *Cx. pipiens* är det färre än hälften som plockar upp viruset, och ännu färre som sedan sprider det vidare.

Eftersom man hittills inte kunnat skilja arterna åt, har de refererats till som *Cx. pipiens/torrentium* vilket står för en okänd potentiell blandning av båda arterna. *Culex pipiens* har världomfattande utbredning och är därför den mest välkända arten, med specifik artkunskap och information tillgänglig från andra världsdelar. Den mindre kända arten, *Cx. torrentium*, är en europeisk art och ytterst lite artspeckifika observationer har gjorts, på grund av dess likhet med *Cx. pipiens*, och den har därför också hamnat i skuggan av sin släkting.

Huvudsyftet med den här avhandlingen är att komma med säker och artspecifik information om de båda myggararterna i Europa, som kan bidra till
bättre förståelse av deras betydelse för spridningen av Sindbisvirus och andra humanpatogena zoonotiska virus med liknande ekologi. I den första uppsatsen utvecklade jag en molekylär metod för att skilja de båda myggaarterna åt i labbet. Metoden baserar sig på genetiska skillnader mellan arterna, och kan utföras på så lite material som enstaka ben av en stickmygga. Denna metod för artidentifiering utgör stommen i alla ytterligare arbeten i denna avhandling.

För den andra och tredje uppsatsen gjorde jag och medarbetare stora fältinsamlingar av Cx. pipiens/torrentium-larver på 49 platser utspridda över Sverige från Torneå i norr, till Kristianstad i söder, samt ytterligare 138 platser i 12 Europeiska länder. Larver från båda arterna hittades ofta tillsammans och var vanligt förekommande i vattentunnor och små vattensamlingar som skapats i traktorskopor, bildäck och dylikt. Totalt 3571 larver artbestämdes sedan med den nyutvecklade molekylära metoden och mycket oväntat visade det sig att den största andelen larver i Sverige (89%) var Cx. torrentium, den minst välkända arten men samtidigt den effektivaste vektor för Sindbisvirus. Culex torrentium fanns över hela landet och var överallt vanligare än Cx. pipiens. Culex pipiens fanns också över hela landet, men blev vanligare längre söderut. Mönstret fortsatte ner i Europa med Cx. pipiens successivt ökade i andel, för att söder om alperna vara den enda av de två arterna som påträffades. I Centraleuropa hittade vi ofta båda arterna tillsammans i jämna proportioner.

I uppsats II artidentifierade jag även 199 vuxna Culex-honor fångade med CDC-fällor som ofta används vid mygginventering. Det visade sig att artsmansättningen bland de vuxna honmyggorna var annorlunda än hos larverna, med mindre andel Cx. torrentium fångade i CDC-fällor. Dock sågs ett liknande mönster med denna insamlingsmetod, med ökande antal Cx. pipiens söderut. Dessutom fann jag att fångsten av oidentifierade Cx. pipiens/torrentium i CDC-fällorna var betydligt större längre söderut i landet. Detta ledde vidare till uppsats IV där jag ville undersöka om fångst med CDC-fällor gav en annorlunda bild, med fler Cx. pipiens, av myggfaunan än larvensamling. Jag jämförde tre olika fälttyper: CDC-fällor, Gravidfällor och ”Ovitraps” som motsvarar larvensamling. Det visade sig att Gravidfällor och Ovitraps gav liknande artfördelningar i procent, medan CDC-fällor jämförelsevis underskattade Cx. torrentium. Det verkar med andra ord som om CDC-fällor inte fångar Cx. torrentium lika bra som Cx. pipiens.

I uppsats V påvisade och isolerade jag virus i artidentifierade Cx. torrentium och Cx. pipiens, vilket aldrig tidigare gjorts. Jag fann att betydligt fler Cx. torrentium var infekterade med Sindbisvirus (36 myggor av 1000), än Cx. pipiens (8 myggor av 1000). Att Sindbisvirus är mycket vanligare hos fältfångade Cx. torrentium, än Cx. pipiens, visar att Cx. torrentium är den mest betydelsefulla enzootiska vektor för Sindbisvirus. Detta stöds också av att Cx. torrentium är så vanlig i området där det förekommer återkommande utbrott av humaninfektioner (papper II).
Att denna avhandling har visat att *Cx. torrentium* är mycket vanlig i norra och mellersta Europa, är ett oväntat resultat som kan vara av generell betydelse för utbredningen av mänskliga sjukdomar orsakade av fågelburna virus. Det är möjligt att Ockelbosjukan, som har *Cx. torrentium* som enzootisk vektor, är beroende av att det finns en tillräckligt stor population *Cx. torrentium*, vilket inte finns i Centraleuropa där viruset påträffats i stickmyggor men där inga fall av sjukdom har rapporterats hos människa. Den påvisade utbredningen av *Cx. torrentium* kan även ha betydelse för spridningen av West Nile virus, som under 2000-talet har fått en ökad betydelse i Europa. Det har ännu inte gjorts några laboratorieexperiment med West Nile virus i *Cx. torrentium*, men resultat från sådana studier skulle i kombination med denna avhandling kunna innebära att risken för utbrott av West Nile virus behöver omvärderas för centrala och norra Europa.
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