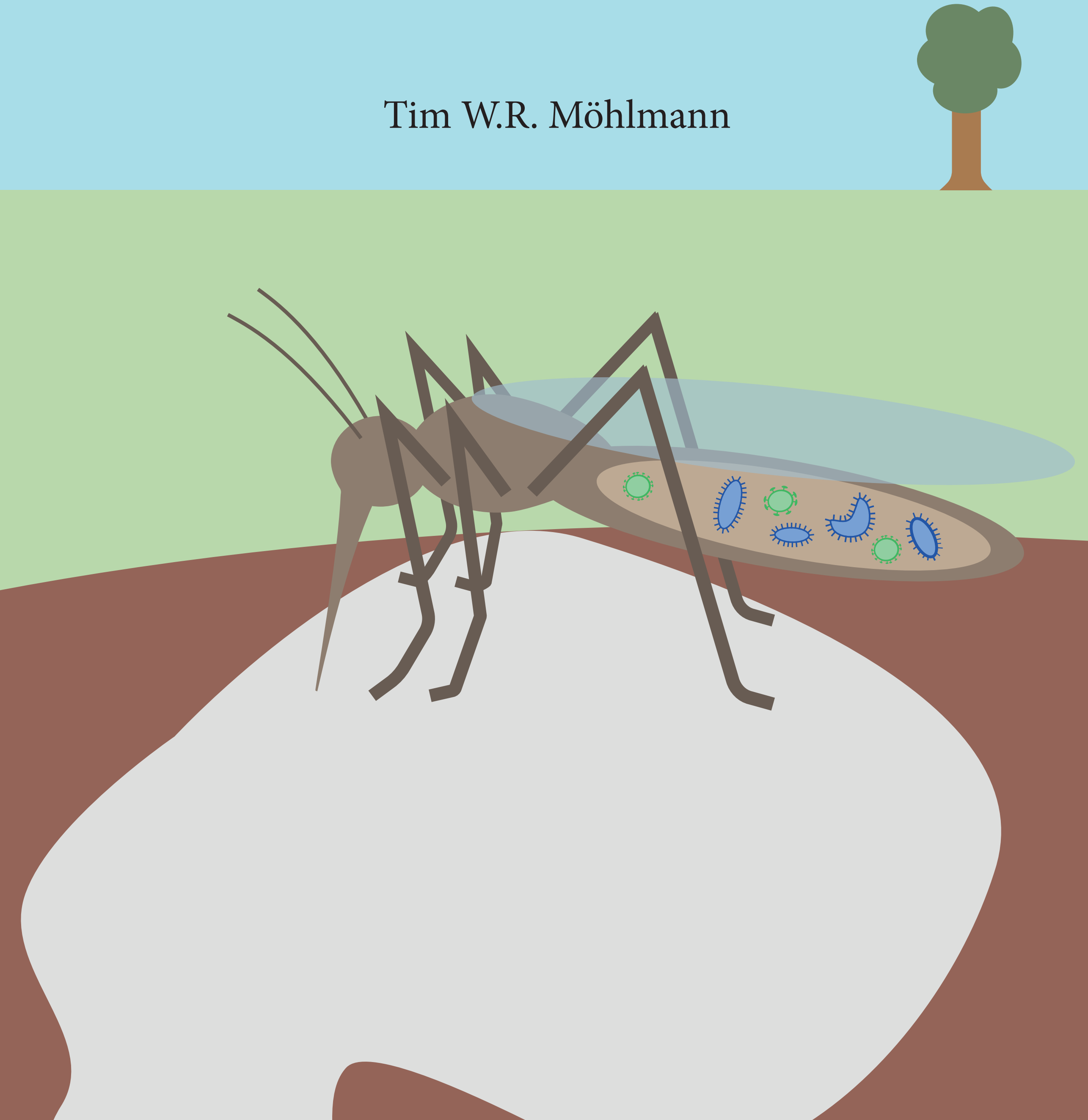
Mosquitoes, midges and microbiota

European vector diversity and the spread of pathogens

Tim W.R. Möhlmann



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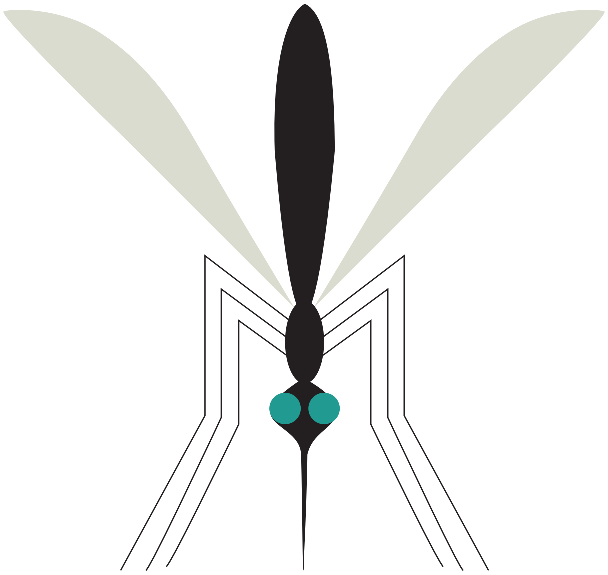

Chapter 1

General introduction

Introduction

A community is an assemblage of organisms found in a certain place and time. Often communities are dominated by a few very common species. However, the composition of a community can change over time (Morin, 2009). Communities occur on a large scale as well as on a more local scale such as an insect community around a livestock farm. Even the smallest organisms such as bacteria and viruses form communities within larger scale environments. The close interaction among species in a community means that a shift in species occurrence within a community can be expected to have an effect on the other species within the same community, as well as the environment or ecosystem that supports this community (Chapin *et al.*, 2000; Ives & Cardinale, 2004; Wang *et al.*, 2011). Both on a large scale, as well as on a small scale, these changes can have a profound impact on the community composition and their effects on the ecosystem at large.

Insects play a crucial role in many ecosystems and the communities therein. With an estimation of several millions of insect species worldwide, they can be found almost everywhere in the world (Stork, 2018). Several insect species have become dependent on blood from other animals for their own reproduction (Lehane, 2005; Shaw *et al.*, 2015). Females of these so-called haematophagous insects take a blood meal from a host to develop their eggs. A female can develop several egg batches during her lifetime and, therefore, has to take multiple blood meals (Lehane, 2005). During a blood meal, the female can take up pathogens that circulate in the host's blood. Some pathogens utilize these blood-feeding insects as a vehicle to get to a new host. Insects that can transmit pathogens in this way are, therefore, referred to as vectors. Some of the well-known insect vectors of human and animal pathogens are mosquitoes, ticks and biting midges (Lehane, 2005).

One Health

With a constantly growing human population, increased travel and trade around the globe and global climate change, we face new challenges such as food security and food safety as well as increased risks of infectious diseases (Purse *et al.*, 2008; Costello *et al.*, 2009; Keesing *et al.*, 2010; Kilpatrick & Randolph, 2012; McMichael, 2013). In a One Health approach, these challenges are tackled with the inclusion of human, veterinary, wildlife, environmental and ecological aspects. These aspects cannot be considered separately if we want to develop durable solutions for these global challenges. For example, to secure sufficient food production for a growing human population, crop production and livestock farms are expanded and intensified. Higher densities of domestic animals can lead to increased risks of animal-disease outbreaks. Often these diseases originate from wildlife populations and are initiated by direct or indirect contacts between wildlife and domesticated animals (Gortázar *et al.*, 2007). To tackle these global problems a One Health approach is key. Prevention of large disease outbreaks in both wildlife and livestock will also protect human health.

Diseases cause major losses in animal production and simultaneously pose a threat to human health due to their zoonotic potential. Over 200 zoonotic diseases that infect both animals and humans are recognized as a threat for animal and human health. Of all emerging infectious diseases, over 60% is zoonotic and of these zoonotic diseases more than 70% originates from wildlife animals (Jones *et al.*, 2008). Vector-borne pathogens are responsible for more than 15% of all emerging infectious diseases and can harm both animals and humans (Jones *et al.*, 2008; WHO, 2017). Combatting vector-borne infectious diseases is, therefore, a perfect example of requiring an transdisciplinary approach due to the complex epidemiology of the diseases, interactions with humans, livestock, wildlife and insect vectors, economic importance, and the multifaceted management of control and prevention (Costello *et al.*, 2009).

Due to increased travel and trade, exotic vector species are repeatedly introduced into new environments. Simultaneously, global rising temperatures provide favourable conditions for these exotic vector species at new locations where the vectors may become established. In addition, increased temperatures accelerate the development of insect vectors, thereby boosting their populations. Finally, higher temperatures increase virus proliferation in the vector, thereby reducing the extrinsic incubation period, which leads to a higher vectorial capacity. These factors all increase the potential of vector-borne pathogen transmission, and consequently increase the risk of disease outbreaks (Kilpatrick & Randolph, 2012).

The global challenges associated with vector-borne diseases need integrated solutions to improve animal and human health. Identification of endemic and exotic vector species, monitoring of vector abundance, predictions of pathogen transmission by vectors, optimization of existing and development of new control methods and testing vector competence of endemic vector species for emerging virus strains will all support preparedness for future disease outbreaks. Only through an integrated approach that involves experts from different scientific disciplines can such solutions be achieved and our preparedness for future disease risks be improved (Garros *et al.*, 2018).

Vectors

Mosquitoes (Culicidae) and biting midges (*Culicoides* spp.) are important vectors of pathogens. Mosquitoes can transmit pathogens such as *Plasmodium*, Zika virus (ZIKV), Chikungunya virus (CHIKV) and West Nile virus (WNV). Biting midges are known to transmit Oropouche virus (OROV), African horse sickness virus (AHSV), Schmallenberg virus (SBV) and bluetongue virus (BTV) (Mellor, 1990; Hubálek & Halouzka, 1999; Rasmussen *et al.*, 2012; Weaver & Lecuit, 2015; Benelli & Romano, 2017; Vogels *et al.*, 2017). Only female mosquitoes and biting midges take blood meals for the development of their eggs. The eggs are deposited in, or close to water or a moist environment. The larvae and pupae of mosquitoes and biting midges have a (semi-)aquatic lifestyle. Larvae of mosquitoes prefer (small) standing water bodies, whereas larvae of biting midges inhabit moist environments rich in organic material, such as mud, tree holes or animal dung (Murray, 1957; Foxi & Delrio, 2010; Steinke *et al.*, 2016). The larvae of both mosquitoes and biting midges are detritivorous or omnivorous and feed on animal- and plant-derived material in their environment (Conte *et al.*, 2007). These are mostly degrading materials, but also include smaller organisms such as algae, bacteria or nematodes. Larvae develop through four instars before they pupate and subsequently hatch into adults after several days. The total development from eggs to larvae, pupae and adults usually takes about 15 to 25 days under optimal conditions, but is largely dependent on food availability and temperature. When temperature drops, development is elongated. During overwintering the development can, therefore, take up to seven months (Benelli *et al.*, 2017).

Adult mosquitoes and biting midges have a terrestrial lifestyle and can move large distances by either active flight or passive movement on wind currents (Ducheyne *et al.*, 2007; Hendrickx *et al.*, 2008; Elbers *et al.*, 2015). Males and females take up sugar-rich substances such as honeydew and nectar as an energy source. In addition, females take blood meals from a host for the development of eggs. For mosquitoes it is known that they show complex search behaviour that assists them in finding a suitable host. Females use carbon dioxide as a long-distance cue for detection of a host. Once in proximity, a combination of heat and body odours produced by the host's skin bacteria helps the females to find their preferred host (Takken, 1991; Bhasin & Mordue, 2001; Mands *et al.*, 2004; Lehane, 2005; Spitzen *et al.*, 2013; Verhulst *et al.*, 2018). Preferred hosts can be humans, large livestock or smaller animals such as birds and even frogs. Each mosquito or biting midge species has its own preference for one or more host species to bite and take a blood meal from (Hubálek & Halouzka, 1999; Mukabana *et al.*, 2002; Lehane, 2005; Garros *et al.*, 2011; Lassen *et al.*, 2012).

The female's biting habit causes nuisance for both animals and humans. Mosquitoes use their proboscis to penetrate the skin and search for a blood vessel to obtain their blood meal. Biting midges employ knife-like mandibles that cut through the skin and create a small pool of blood from which they can feed (Robinson, 1939; McKeever *et al.*, 1988). Both mosquitoes and biting midges release anticoagulation compounds to facilitate the uptake of blood from their host. These compounds, released with the saliva of the vector during their bite, can trigger a response in the host body that causes the place of the bite to itch (Ribeiro & Francischetti, 2003). In some cases this can result in an allergic reaction, as is the case for insect-bite hypersensitivity in horses which is caused by components of the saliva of biting midges (Hellberg *et al.*, 2006). Besides the nuisance, transmission of pathogens by these vectors has a more profound impact on the health of animals and humans.

Although mosquitoes and biting midges have many features in common, they also exhibit important differences. These differences become apparent in their larval habitats and biting behaviour as mentioned above, but also in other aspects such as the pathogens they transmit and their lifespan. Biting midges mainly transmit viruses, whereas mosquitoes are also able to transmit malaria parasites and bacteria that can cause diseases. Finally, adult mosquitoes can live for several months whereas adult biting midges often survive only several weeks (Wittmann *et al.*, 2002; Xue *et al.*, 2010).

Pathogens

Globally, hundreds of millions of people get infected with a vector-borne pathogen every year. About 700,000 persons die due to these infections thereby accounting for an estimated 17% of the global mortality due to infectious diseases (WHO, 2017). The malaria parasite *Plasmodium falciparum* remains the most deadly pathogen transmitted by mosquitoes and causes disease mainly in sub-Saharan Africa. Additionally, mosquito-transmitted viruses such as dengue virus, CHIKV, WNV and ZIKV cause severe diseases in both Asia and the Americas. Biting midges are currently known to transmit only one human pathogen, Oropouche virus, of which the distribution is limited to south- and central-America. This virus has caused clinical cases in Brazil, Peru, Panama, Trinidad and Tobago, but has not spread outside of Latin America so far (Sakkas *et al.*, 2018).

After the elimination of malaria, the European continent has remained relatively free from mosquito-borne pathogens. However, from the mid-1990's Europe has seen endemic outbreaks with human cases of WNV in the Balkan area (Romania, Greece, Croatia, Serbia, Montenegro, Hungary) and southern Europe (Spain and Italy). The largest outbreak occurred in Bucharest in 1996 with 293 hospitalized cases, which resulted in 17 deaths (Hubálek & Halouzka, 1999; Calistri *et al.*, 2010; Sambri *et al.*, 2013; ECDC, 2016). For the year 2018 the numbers of WNV cases reported to European health authorities are higher than ever before (ECDC, 2018). In addition, outbreaks of autochthonous human CHIKV cases occurred in Italy in 2007 with 205 cases and one death, and in France in 2010 and 2014 with 12 confirmed cases (Rezza *et al.*, 2007; Grandadam *et al.*, 2011; Delisle *et al.*, 2015). With increased travel and trade around the world and globally increasing temperatures there is a growing concern that more vector-borne pathogens will make their way into Europe (Schaffner *et al.*, 2009). Being prepared for potential spread of pathogens is the first step to limit the fast spread of a pathogen and thereby reduce the incidence of diseases.

In addition to human casualties, both wildlife and livestock are affected by vector-borne pathogens (Mellor *et al.*, 2000; Buckley *et al.*, 2003; Van Schaik *et al.*, 2008; Saegerman *et al.*, 2014; Veldhuis *et al.*, 2014). Many bird species can become infected by WNV and Usutu virus, which are mosquito-transmitted viruses. Arthropod-borne pathogens that affect large livestock are most often transmitted by *Culicoides* biting midges. African horse sickness virus and BTV have been a serious problem in African countries for many years (Tabachnick, 2003; Carpenter *et al.*, 2017). In Europe, biting midge-borne diseases were not a serious issue until 2006. However, since the first introduction of BTV in 2006, and the outbreak of SBV in 2011, both diseases have been enzootic in Europe (Wernike *et al.*, 2012; Gubbins *et al.*, 2014). When animals get infected with these viruses, it often leads to reduced milk production. In addition, when the mother becomes infected with these viruses during gestation this can result in non-viable calves and lambs. These impacts, in addition to livestock movement restrictions, resulted in considerable economic losses (Mellor *et al.*, 2000; Van Schaik *et al.*, 2008; Saegerman *et al.*, 2014; Veldhuis *et al.*, 2014).

In the beginning of this century, mosquito-transmitted pathogens such as WNV, CHIKV and ZIKV were neglected viruses with relatively limited impact. However, all three arboviruses have quickly spread throughout immunologically naïve populations (Mayer *et al.*, 2017). The massive outbreaks of these viruses came as a surprise, although smaller outbreaks in previously unaffected areas could have been recognised as early warning signals. Biting midge-borne pathogens such as AHSV and Shuni virus (SHUV) are currently known to circulate in some parts of the world but have thus far not entered the European continent (Tabachnick, 2003; van Eeden *et al.*, 2014; Golender *et al.*, 2015; Golender *et al.*, 2016; Carpenter *et al.*, 2017). Their introduction into Europe could result in a quick spread of these diseases throughout the continent, similar to the examples of WNV, CHIKV or ZIKV in other parts of the world. To increase human health and animal welfare and to prevent unnecessary economic losses due to rapid disease outbreaks, it is essential to identify the (potential) vectors that transmit these pathogens.

Vector communities

The first step to assess the risk for vector-borne disease spread is obtaining knowledge about which vector species occur in a specific area, and what their potential is to transmit pathogens. To identify which species are present in a specific area, monitoring of the vectors is necessary. For monitoring of species and their abundance, mosquitoes can be caught with mosquito traps that lure the mosquitoes with the use of CO_2 and/or odours and actively trap them with the use of a fan (Kitau *et al.*, 2010; Hiscox *et al.*, 2014; Luhken *et al.*, 2014). Similarly, biting midges can also be caught with a trap, although attraction of biting midges is more efficient with a black-light than with CO_2 or odours (Venter *et al.*, 2009). Collection of mosquito and biting midge vectors is most efficient from dusk to dawn because the majority of vector species is active during twilight. However, a 24-hour collection will also catch vectors that are day-active. For a full inventory of species diversity, monitoring should therefore include day trapping and should be continued throughout the year, because not all vector species have a similar phenology (Ander *et al.*, 2012).

Multiple (surveillance) studies have collected mosquitoes and biting midges in Europe (Schäfer & Lundström, 2001; Schäfer *et al.*, 2004; Balenghien *et al.*, 2006; Rettich *et al.*, 2007; Fassotte *et al.*, 2008; Osório *et al.*, 2008; Kiel *et al.*, 2009; Chaves *et al.*, 2011; Kaufmann *et al.*, 2012; Venail *et al.*, 2012; González *et al.*, 2013; Lundström *et al.*, 2013; Versteirt *et al.*, 2013; Ibanez-Justicia *et al.*, 2015; Roiz *et al.*, 2015; Sohier *et al.*, 2018). Several European mosquito species, including the *Culex* (*Cx.*) *pipiens* complex, *Cx. modestus* (Ficalbi 1889), the *Anopheles* (*An.*) *maculipennis* complex, *Aedes (Ae.) vexans* (Meigen 1830) and *Ae. albopictus* (Skuse 1895) are known to be vectors of parasites or viruses such as *Plasmodium*, Rift Valley fever virus, ZIKV or WNV (Han *et al.*, 1999; Balenghien *et al.*, 2006; Chevalier *et al.*, 2010; Aliota *et al.*, 2016). Similarly, biting midges from the Obsoletus group (*C. chiopterus, C. dewulfi, C. montanus, C. obsoletus* s.s. and *C. scoticus*) (De Liberato *et al.*, 2005; Savini *et al.*, 2005;

Carpenter *et al.*, 2008; Elbers *et al.*, 2013; Koenraadt *et al.*, 2014; Meiswinkel *et al.*, 2014), *C. imicola* (Dzhafarov 1963) (Goffredo & Meiswinkel, 2003), *C. pulicaris* (Linnaeus 1758) (Purse *et al.*, 2004; Takken & Knols, 2007; Koenraadt *et al.*, 2014), and *C. punctatus* (Meigen 1804) (Takken & Knols, 2007; Carpenter *et al.*, 2009; Hoffmann *et al.*, 2009; Wilson & Mellor, 2009; Ander *et al.*, 2012; Balenghien *et al.*, 2014; Meiswinkel *et al.*, 2014) are known to act as vectors of BTV and SBV in Europe.

European studies on mosquito and biting midge species diversity have often focussed on a specific habitat, region or country (Schäfer & Lundström, 2001; Schäfer *et al.*, 2004; Balenghien *et al.*, 2006; Rettich *et al.*, 2007; Fassotte *et al.*, 2008; Osório *et al.*, 2008; Kiel *et al.*, 2009; Chaves *et al.*, 2011; Kaufmann *et al.*, 2012; Venail *et al.*, 2012; González *et al.*, 2013; Lundström *et al.*, 2013; Versteirt *et al.*, 2013; Ibanez-Justicia *et al.*, 2015; Roiz *et al.*, 2015; Sohier *et al.*, 2018). Although information on the occurrence of vector species in specific regions is highly valuable, it is challenging to compare results from these studies across Europe. Standardized cross-European studies would help to identify which species are found only in specific regions and which species occur throughout Europe. The identification of a core community of vectors could help to focus on specific species that have the potential of rapidly spreading a pathogen throughout the continent. In addition, such an inventory can support mathematical models to predict the impact of vector diversity and abundance on patterns of disease spread.

The spread of pathogens and subsequently disease is linked to the diversity of vector species in a community and this diversity can either increase or decrease disease risk (Keesing *et al.*, 2006). A theoretical modelling study by Roche *et al.* (2013) suggested that higher vector species richness can increase pathogen transmission. This enhanced pathogen transmission was mostly induced by an increased total vector abundance that was directly linked to species richness. On the other hand, a study by Chaves *et al.* (2011) suggested that higher diversity in vector communities decreases mosquito abundance, and consequently also reduces the risk of amplification and spread of disease. Increased total vector abundance seems to be the defining factor for enhanced pathogen transmission, as well as for epidemic take-off, even if the vector species have a low vector competence (Roche *et al.*, 2013). Linking vector capture data with environmental factors and subsequently modelling vector abundance and their influence on disease spread are fundamental. To better understand the role of vector communities in the spread of diseases, knowledge about vector species distribution, abundance, and richness is therefore essential.

Diversity measures

Species richness refers to the number of species in a given area or in a given sample, and does not take the abundance of the species into account. The rarity or commonness of species in a community is therefore not evaluated with species richness. Species diversity, on the other hand, includes both the number of species and the distribution of individuals among those species. Species diversity should only be used in the context of a diversity index, which includes a relation between the species richness and the number of individuals of each species (Spellerberg & Fedor, 2003; Morin, 2009). Several indices of diversity are used and account for the relation between species richness and species abundance in different ways. A commonly used diversity index is the Shannon-Wiener Index. This index takes into account how many species there are (species richness) and additionally accounts for the evenness of the distribution of individuals over species (abundance) (Spellerberg & Fedor, 2003).

Another measure of diversity that is often used in ecological research is the Simpson Diversity Index (Simpson, 1949). This measure also accounts for species richness and species abundance. It gives the probability that two individuals taken at random from a dataset are of the same species (Hunter & Gaston, 1988). This measure gives more weight to abundant species and addition of rare species causes only limited changes in the diversity index.

By using these diversity indices, a comparison can be made among groups of samples based on factors such as habitat or country. The indices can be used regardless of the identity of the individuals and are useful for calculation of mosquito or biting midge diversity, as well as of bacterial community diversity. Ultimately, it might be possible to use these measures to predict the risk of pathogen spread, as was suggested by studies that linked diversity measures to the increase or decrease of pathogen transmission (Keesing *et al.*, 2006; Chaves *et al.*, 2011; Roche *et al.*, 2013).

Species groups and complexes

Most mosquito and biting midge species can be identified using morphological characteristics (Becker *et al.*, 2010; Mathieu *et al.*, 2010; Mathieu *et al.*, 2012). However, in the literature some species are clustered in a cladistically artificial species group or complex. A species group refers to those species that are related or morphologically similar, while still exhibiting a notable degree of morphological differentiation, whereas a species complex includes isomorphic species which cannot currently be differentiated morphologically (Harrup *et al.*, 2015). Identification of species within a species group or complex can be extremely relevant because the species can exhibit different behaviour in, for example, breeding habitat selection or host preference. These behavioural differences may lead to different contributions to pathogen transmission. Currently, identification of species within a group or complex is often neglected because it can be laborious and time consuming (De Liberato *et al.*, 2005; Conte *et al.*, 2007; Elbers & Meiswinkel, 2014; Foxi *et al.*, 2016; Versteirt *et al.*, 2017). This makes comparisons of outcomes among studies difficult and, therefore, it remains uncertain to what extent specific species contribute to pathogen transmission.

In Europe, *Culex (Cx.) pipiens* is the most widespread and abundant mosquito species and the main vector of WNV (Fonseca *et al.*, 2004; Kilpatrick *et al.*, 2005). The *Cx. pipiens* complex consists of several closely related species and biotypes of which only the species *Cx. pipiens* (Linnaeus 1758) occurs in Europe (Farajollahi *et al.*, 2011). *Culex pipiens* consists of two morphologically similar biotypes, named *pipiens* (Linnaeus 1758) and *molestus* (Forskål 1775) which show distinct behaviour in winter activity, reproduction and host preference. The hybrid forms of the two biotypes show intermediate behaviour (Marshall & Staley, 1936; Shute, 1941; Willcox, 1941; Sanburg & Larsen, 1973; Byrne & Nichols, 1999; Dohm & Turell, 2001; Fonseca *et al.*, 2004; Fritz *et al.*, 2015).

Species within the Obsoletus group are considered the most important *Culicoides* biting midge vectors of animal pathogens in central and northern Europe (Elbers *et al.*, 2015). Obsoletus group species occur throughout Europe and the group consists of at least five related species, namely *C. chiopterus* (Meigen, 1830), *C. dewulfi* (Goetghebuer, 1936), *C. montanus* (Shakirzjanova, 1962), *C. obsoletus* sensu stricto (s.s.) (Meigen, 1818) and *C. scoticus* (Downes & Kettle, 1952) (Goffredo *et al.*, 2016). Three species within the Obsoletus group together form the Obsoletus complex: *C. obsoletus* s.s., *C. scoticus* and *C. montanus* (Harrup *et al.*, 2015). All of the Obsoletus group species are associated with livestock, although each species has a distinct preference for breeding habitat and host (Harrup *et al.*, 2013; Viennet *et al.*, 2013; Zimmer *et al.*, 2014; Elbers & Meiswinkel, 2015; Zimmer *et al.*, 2015; Steinke *et al.*, 2016). In addition, SBV infection rate in the field was significantly different among these species, with the highest infection rates detected in *C. scoticus* (0.43%) and no virus-positive individuals detected for *C. dewulfi* (Elbers *et al.*, 2013).

The two biotypes of *Cx. pipiens* and species of the Obsoletus group are currently considered the most important vector species in Europe. It is therefore important to understand what role a specific species within these species groups can play in pathogen transmission. The relative distribution of species within these groups remains under-investigated, even though ecological and behavioural differences suggest that we cannot consider them as one mosquito or biting midge group.

Vector competence

Viruses can be horizontally transmitted by vectors from one vertebrate host to the other if they are picked up during a blood meal. Before transmission to a new host, the virus has to overcome several barriers in the insect's body to be successfully transmitted (Fig. 1). Once a virus has entered the gut of a mosquito or biting midge, it first has to infect the midgut epithelial cells. This first step is considered a major barrier for the virus particles (Fu *et al.*, 1999; Mills *et al.*, 2017). Inside the midgut cells, proliferation of the virus can take place. Once the virus has escaped from the midgut cells, it has overcome the infection barrier and can disseminate throughout the insect body and infect other tissues in order to amplify to higher virus titers. However, at this stage the virus has to cope with the insect's cellular responses and immune system in the haemolymph, which is referred to as the dissemination barrier (Fu

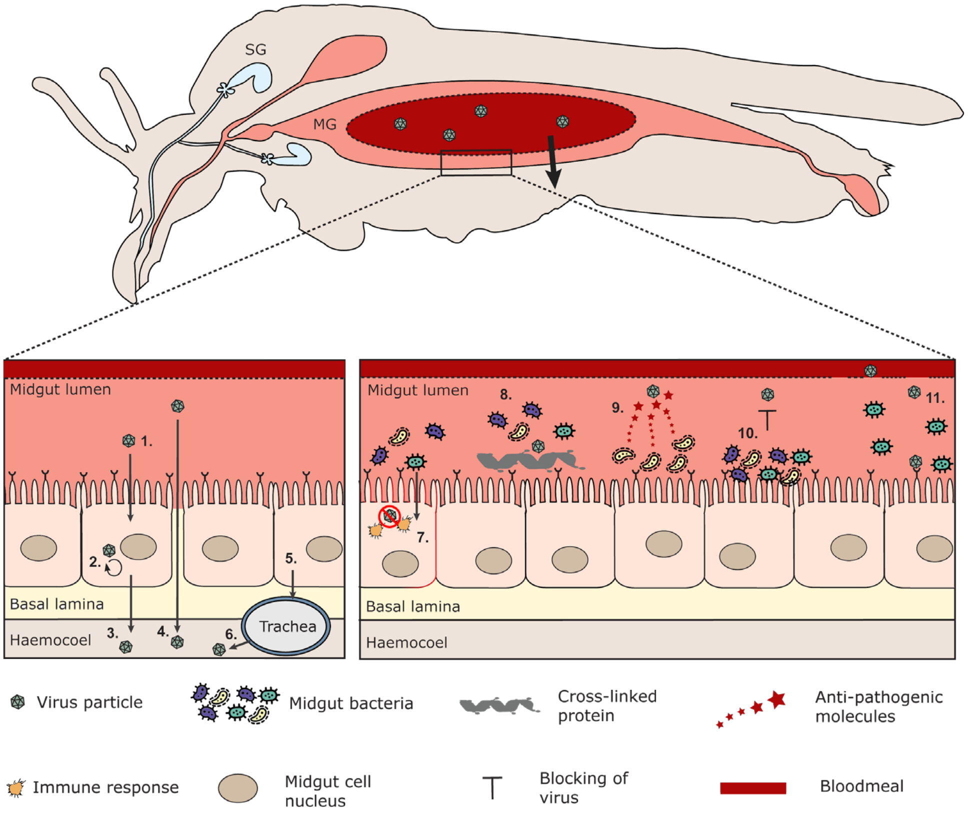
SG
MG
Midgut lumen
1.
2.
5.
Basal lamina
Haemocoel
3.
4.
6.
Trachea
Midgut lumen
8.
9.
10.
11.
7.
Basal lamina
Haemocoel
Virus particle
Midgut bacteria
Cross-linked protein
Anti-pathogenic molecules
Immune response
Midgut cell nucleus
Blocking of virus
Bloodmeal

Figure 1. Schematic overview of barriers to arbovirus infection in biting midges. Schematic longitudinal cross-section of a *Culicoides* biting midge. Arrow indicates the passage of virus particles through the midgut (MG) barrier. A salivary gland (SG) barrier is thought to be non-existent in biting midges (Fu *et al.*, 1999; Mellor *et al.*, 2000). The dashed circle in the midgut represents the peritrophic membrane. Left inset: (1) Infection of midgut epithelial cells via binding to a putative receptor protein. (2) Virus replication in midgut epithelial cells. (3) Release of virus via budding from midgut epithelial cells and direct passage through the basal lamina into the haemocoel. (4) Direct virus passage into the haemocoel through a 'leaky' midgut (Mellor *et al.*, 2000). (5) Virus infection of trachea after budding from midgut epithelial cells. (6) Budding of virus from the trachea into the haemocoel. After passage into the haemocoel, the virus will infect secondary target organs including the salivary glands (Fu *et al.*, 1999). Right inset: (7) Midgut bacteria can stimulate an antimicrobial immune response that also negatively affects virus infection. (8) A layer of cross-linked proteins that inhibits over-activation of the immune system by bacteria also reduces interaction of virus particles with midgut epithelial cells (Kumar *et al.*, 2010). (9) Specific midgut bacteria can secrete anti-pathogenic molecules, such as reactive oxygen intermediates or secondary metabolites, that negatively affect pathogens in the midgut (Cirimotich, Dong, *et al.*, 2011). (10) Bacterial populations may provide a physical barrier to virus interaction with midgut epithelial cells, consequently hindering infection. (11) Specific midgut bacteria enhance the production of proteins, which inhibit the formation of a peritrophic membrane and biofilm formation by bacteria, thereby facilitating virus infection (Cabezas-Cruz *et al.*, 2016; Abraham *et al.*, 2017). Figure adapted from Megahed, (1956), Cirimotich, *et al.*, (2011), and Vogels *et al.*, (2017).

et al., 1999). After amplification in the insect body, the virus can infect the salivary glands. When virus is present in the saliva of the vector, virus particles will be released into a new host during a next blood meal. In the case of mosquitoes there is a salivary gland barrier that limits virus spread, whereas a salivary gland barrier is thought to be absent in biting midges (Fu *et al.*, 1999; Mellor *et al.*, 2000; Vogels *et al.*, 2017). In addition to horizontal transmission of pathogens, i.e. from insect to host and *vice versa*, some mosquito-borne viruses can be vertically transmitted to their offspring, whereas this phenomenon is thought to be absent in biting midges (Mellor *et al.*, 2000; Lequime *et al.*, 2016)

Each of the barriers that has an effect on successful virus transmission influences vector competence. Both laboratory-reared and field-collected vectors show inter- and intra-species variability in vector competence. Only a proportion of individuals is likely to become infected after oral exposure to a specific arbovirus (Tabachnick, 1991). Some of the traits that affect vector competence have a genetic background and have been shown to be heritable (Tabachnick, 1991; Fu *et al.*, 1999). In addition to genetic differences among individuals, extrinsic factors such as temperature and larval development conditions can influence vector competence (Mellor *et al.*, 2000; Mills *et al.*, 2017). More recently, the gut microbiome of vectors was hypothesized to play an important role in virus infection and replication (Fig. 1). It is suggested that gut bacteria can interact with virus infection either through the activation of the vector immune response, formation of a physical barrier of cross-linked protein layers by the gut cells, or the production of compounds by bacteria that directly interfere with viruses entering the vector's gut (Fig. 1) (Kumar *et al.*, 2010). Several species of bacteria have been found to influence vector competence for specific pathogens by mosquitoes (Durvasula *et al.*, 1997; Azambuja *et al.*, 2004; Azambuja *et al.*, 2005; Favia *et al.*, 2007; Xi *et al.*, 2008; Bisi & Lampe, 2011; Iturbe-Ormaetxe *et al.*, 2011; Apte-Deshpande *et al.*, 2012; Ramirez *et al.*, 2012; Apte-Deshpande *et al.*, 2014; Dodson *et al.*, 2014; Ramirez *et al.*, 2014; Stathopoulos *et al.*, 2014; Barletta *et al.*, 2017; Wang *et al.*, 2017; Guégan *et al.*, 2018).

Microbial communities

Vector community composition and species diversity can have an impact on pathogen transmission. However, pathogen transmission also depends on the interactions among the pathogen, the vector, and its host. In all of these interactions the community of associated microorganisms plays an important role (Guégan *et al.*, 2018).

All insects harbour a diversity of microbes on the outside and inside of their body and this microbiota can account for up to 10% of the total insect's biomass (Douglas, 2015). The gut system is one of the most accessible bacterial habitats of an insect. For these bacteria the gut habitat provides access to nutrients and protection against external factors such as desiccation and radiation. However, the gut of insects also represents a harsh environment with unfavourable physiochemical conditions such as fluctuating pH levels and reactive oxygen, as well as compounds produced by the insect immune response and changes in or loss of habitat because of metamorphosis during the insect's development (Douglas, 2015). Bacteria that inhabit the gut system can increase the fitness of insects by providing essential amino acids and vitamins or by protection against pathogens (Gündüz & Douglas, 2009; Brune, 2014; Frago *et al.*, 2017; Correa *et al.*, 2018; Valzania *et al.*, 2018). It is evident that bacteria and other microorganisms have a substantial impact on their insect host. Revealing the insect-associated microorganisms throughout the insect lifecycle is necessary to identify their role in insect fitness and interaction with pathogens.

Species interactions

The interaction of mosquitoes and biting midges with microorganisms is apparent throughout their lifecycle. These interactions involve microorganisms associated with the insect as well as those in their environment. For example, female mosquitoes are known to select suitable breeding sites based on odours released by specific bacteria present in a habitat (Takken, 1999; Eneh *et al.*, 2018). Through deposition of eggs in the habitat, female mosquitoes might inoculate the water with their associated microorganisms (Lindh *et al.*, 2008). The larvae that emerge feed on degrading material but also on algae, bacteria and nematodes (Merritt *et al.*, 1992; Conte *et al.*, 2007). Subsequently, bacteria in the larval habitat can influence life-history traits such as pupation rate and adult body size. These traits are likely influenced by bacteria through their role in nutrition availability or interaction with the larval metabolism, thereby underpinning the importance of these interactions (Dickson *et al.*, 2017). Furthermore, larvae of mosquitoes have been shown to cannibalize their own siblings, as well as predate on larvae of other mosquito species (Merritt *et al.*, 1992; Koenraadt & Takken, 2003). This direct interaction among larvae of different mosquito species has consequences for the adult vector community and subsequently for patterns of disease spread.

Adult mosquitoes and biting midges also interact with bacteria, as well as with mammalian hosts and viral pathogens. Adult female vectors are largely dependent on odours for the localisation of a suitable host for a blood meal (Zwiebel & Takken, 2004). These odours are produced by specific skin bacteria and can be either attractive or repellent to mosquitoes or biting midges (Verhulst *et al.*, 2010; Takken & Verhulst, 2013; Verhulst *et al.*, 2018). The attractiveness of hosts can in turn be manipulated by pathogens as was shown for malaria parasites that indirectly affect the odour-blend composition excreted by humans (Busula *et al.*, 2017). Because of their blood-feeding habit, mosquitoes and biting midges are capable of transmitting pathogens to a range of vertebrate hosts. These pathogens can cause disease and this sometimes lead to death of the host. Indirectly the insect vectors thereby affect their surrounding environment. When communities of large grazers are affected by the pathogens, this will have an influence on the vegetation in the area and subsequently on the ecosystem as a whole (Olff & Ritchie, 1998; Knapp *et al.*, 1999). Changes in the ecosystem feed back on the insect vectors by creating new (breeding) habitats. Rapid adaptation to new conditions in the ecosystem might be facilitated by the close interactions of the insect vector and their gut microbes (Minard *et al.*, 2013).

Gut bacteria not only interact with their vector host, but may also interact with pathogens that are transmitted by these vectors and can either increase or decrease pathogen infection (Azambuja *et al.*, 2004; Azambuja *et al.*, 2005; Xi *et al.*, 2008; Cirimotich, *et al.*, 2011; Apte-Deshpande *et al.*, 2012; Ramirez *et al.*, 2012; Apte-Deshpande *et al.*, 2014; Ramirez *et al.*, 2014; Hegde *et al.*, 2015; Barletta *et al.*, 2017). This underscores the importance and consequences of multispecies interactions. It shows that all interactions among bacteria, pathogens, vectors, hosts and their environment should be considered within a One Health approach when investigating the effect of vectors on disease spread.

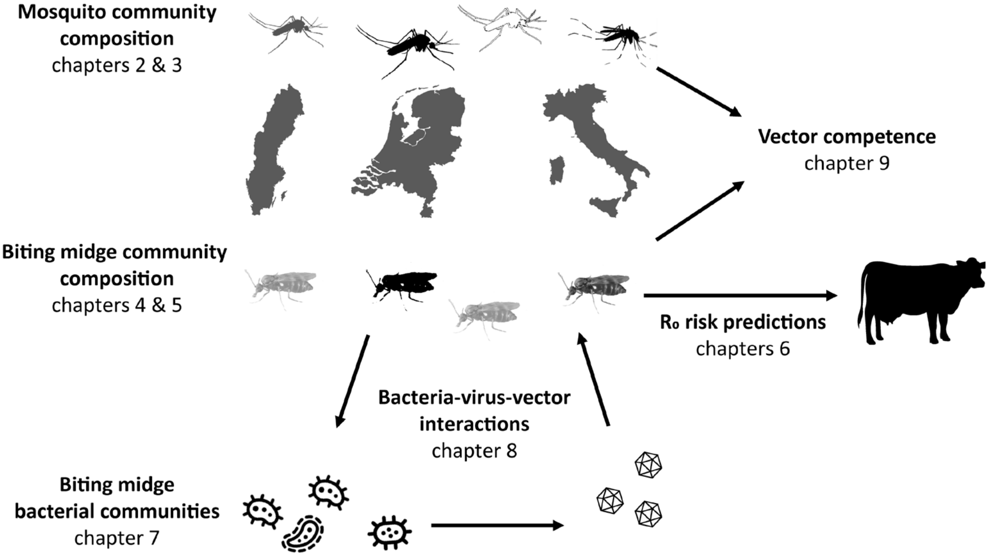
Mosquito community composition
chapters 2 & 3
Vector competence
chapter 9
Biting midge community composition
chapters 4 & 5
R₀ risk predictions
chapters 6
Bacteria-virus-vector interactions
chapter 8
Biting midge bacterial communities
chapter 7

Figure 2. Graphical overview of the chapters in this PhD thesis. This thesis presents the community composition of mosquitoes and biting midges in Sweden, The Netherlands, and Italy in chapters 2 and 4. The biotypes and hybrids of *Culex pipiens* mosquitoes (*Cx. p. pipiens, Cx. p. molestus* and their hybrids) in chapter 3, and four biting midge species in the Obsoletus group (*C. chiopterus, C. dewulfi, C. obsoletus* s.s., *C. scoticus*) in chapter 5. This is followed by risk predictions of bluetongue transmission throughout Europe based on biting midge abundance and biting rate in chapter 6. The bacterial communities in field-collected species and different life stages of laboratory-reared biting midges is presented in chapter 7. The thesis continues with the influence of bacterial communities in mosquitoes and biting midges on virus infection in chapter 8. Finally, the vector competence of mosquitoes and biting midges for transmission of Shuni virus is described in chapter 9.

Aims of this thesis

The aims of this thesis were fivefold and include the identification of mosquitoes and biting midges species community composition in different habitats at three latitudes in Europe. Furthermore it includes the determination of bacterial communities throughout the lifecycle of biting midges, as well as the gut bacterial community composition in multiple field-collected and laboratory-reared biting midge species. The thesis continues to elucidate how these gut bacterial communities influence virus infection in mosquitoes and biting midges. Additionally, it describes how we investigated if endemic or exotic mosquito and biting midge species are competent vectors for a potential zoonotic virus. Finally, the obtained results of European vector community composition and their associated gut bacterial community is used to predict disease risk based on mathematical models. This facilitates our preparedness for vector-borne infectious diseases using a One Health approach.

With a focus on Europe, this research aims to answer the questions (1) whether environmental and geographical aspects influence vector abundance and community composition of both the vectors and their bacterial community, (2) if field collections of biting midges can be used to predict risks for vector-borne veterinary diseases, (3) if gut bacteria can influence pathogen infection and transmission in mosquitoes and biting midges and (4) whether endemic and exotic mosquito and biting midge species are competent vectors of the potentially zoonotic Shuni virus.

Outline of the thesis

This thesis starts with presenting the investigations of the composition of mosquito and biting midge vector species in Europe, and how habitat type or geographic distance among locations influence the communities of these vectors. **Chapter 2** addresses the diversity of mosquito species in different habitat types and at different latitudes in Europe, with sampling locations in Sweden, The Netherlands and Italy. The number of species and individuals found in farm, peri-urban and wetland habitats for each of the three countries are compared using species diversity indices and statistical ordination techniques.

Culex pipiens is the most abundant mosquito species found across Europe and consists of two biotypes and their hybrid forms. These biotypes and hybrids are differentially attracted to birds or mammals. Understanding the factors that drive their relative abundance can therefore be key for better disease risk predictions. To answer the question if the two biotypes and their hybrids occur in similar distribution throughout Europe, **Chapter 3** shows the effects of habitat type and latitude on the relative abundance of the *Culex pipiens* biotypes and their hybrid form at different sampling locations across Europe.

In addition to mosquitoes, biting midges are also responsible for the transmission of pathogens throughout Europe. To identify factors that influence biting midge communities,

Chapter 4 presents an investigation if the diversity of biting midge species is associated with habitat type and latitude at different sampling locations across Europe. These locations were identical to those used for collection of mosquitoes as discussed in Chapter 2. The biting midge community at farm, peri-urban, and wetland habitats in Sweden, The Netherlands and Italy are compared with species diversity indices and statistical ordination techniques.

The Obsoletus group consists of at least five biting midge species (*Culicoides chiopterus, C. dewulfi, C. montanus, C. obsoletus* s.s. and *C. scoticus*) that differ in host preference and infection rate for pathogens. Knowledge about each species' distribution is therefore essential. To do so, **Chapter 5** addresses the question if habitat type or latitude influences the relative abundance of species in the Obsoletus group across Europe. Identification was performed with molecular tools for Obsoletus group biting midges from farm, peri-urban and wetland habitats in Sweden, The Netherlands and Italy. Pointing out the most important vector species around livestock farms may be crucial for predictions of pathogen transmission throughout Europe.

Based on our biting midge captures from the field (Chapter 4), the risk of BTV disease outbreaks in Europe can be computed. A predictive model for BTV transmission throughout Europe was therefore developed and validated in **Chapter 6**. Biting midge catch dynamics are predicted using environmental factors such as temperature, rainfall and humidity. The model further integrates the field-collection of biting midges with bluetongue prevalence amongst sentinel cattle from the 2007 outbreak in The Netherlands. Based on the biting midge abundance model in combination with the bluetongue prevalence, an inferred biting rate per cow per day based on the daily biting midge catch predictions is presented in this chapter. Finally, we show how these predictions can be used to estimate BTV transmission dynamics for different seasons and years across Europe.

Vectors show inter- and intraspecific variability in vector competence and only a proportion of individuals becomes infected after exposure to an arbovirus. The gut microbiome of vectors is hypothesized to be an important factor in differences of infection rates. **Chapter 7** addresses the questions whether the internal microbiota of biting midges changes during their lifecycle and what the variation of gut bacterial communities is among adult females of multiple species collected in the field (Chapters 4 and 5). We investigated the bacterial community of eggs, larvae, pupae and adults of two laboratory-reared biting midge species. Furthermore, we compare the gut bacterial community of laboratory-reared and field-collected adult females. Finally, we investigated the influence of biting midge species as well as geographic distance on the gut bacterial community composition of field-collected individuals.

The differences found in gut bacterial communities among several biting midge species support the hypothesis that these bacteria may interfere with pathogen infection. We therefore wanted to know the effect of midgut bacteria on arbovirus transmission. In **Chapter 8**, I present the effects of midgut microbiota of mosquitoes and biting midges on the infection capacity of different viruses. Two laboratory-reared biting midge species were treated with antibiotics and subsequently exposed to Schmallenberg virus. In addition, a laboratory-reared mosquito species was exposed to Zika virus or Chikungunya virus after antibiotic treatment. Infection rates are compared between the antibiotic-treated and untreated mosquitoes and biting midges.

Preparedness for potential disease outbreaks is part of the One Health strategy. Therefore, **Chapter 9** addresses the question whether mosquitoes and biting midges can transmit Shuni virus. Literature suggested that mosquitoes as well as biting midges are potential vectors of Shuni virus. This chapter presents the results of infection experiments with two mosquito and two biting midge species for this virus. The four potential vector species were orally exposed to an infectious blood meal. Subsequently, infection rates as well as dissemination and transmission efficiencies were determined to elucidate if this highly pathogenic virus with extremely broad tropism and zoonotic potential can be efficiently transmitted by mosquitoes and biting midges.

The concluding **Chapter 10** integrates the results of the different chapters and discusses how this knowledge can be used to improve our preparedness for future disease outbreaks. The question will not be if, but when the next outbreak of a vector-borne disease will occur. Monitoring and identification of vectors can help to inform mathematical models that predict areas most at risk of disease outbreaks. Based on these predictions, control measures can be implemented, which can include new control tools based on bacteria to reduce virus infection in vectors. Future research should integrate knowledge about the pathogen, its vector, and hosts to better understand their interactions and consequences for disease outbreaks.

Acknowledgements

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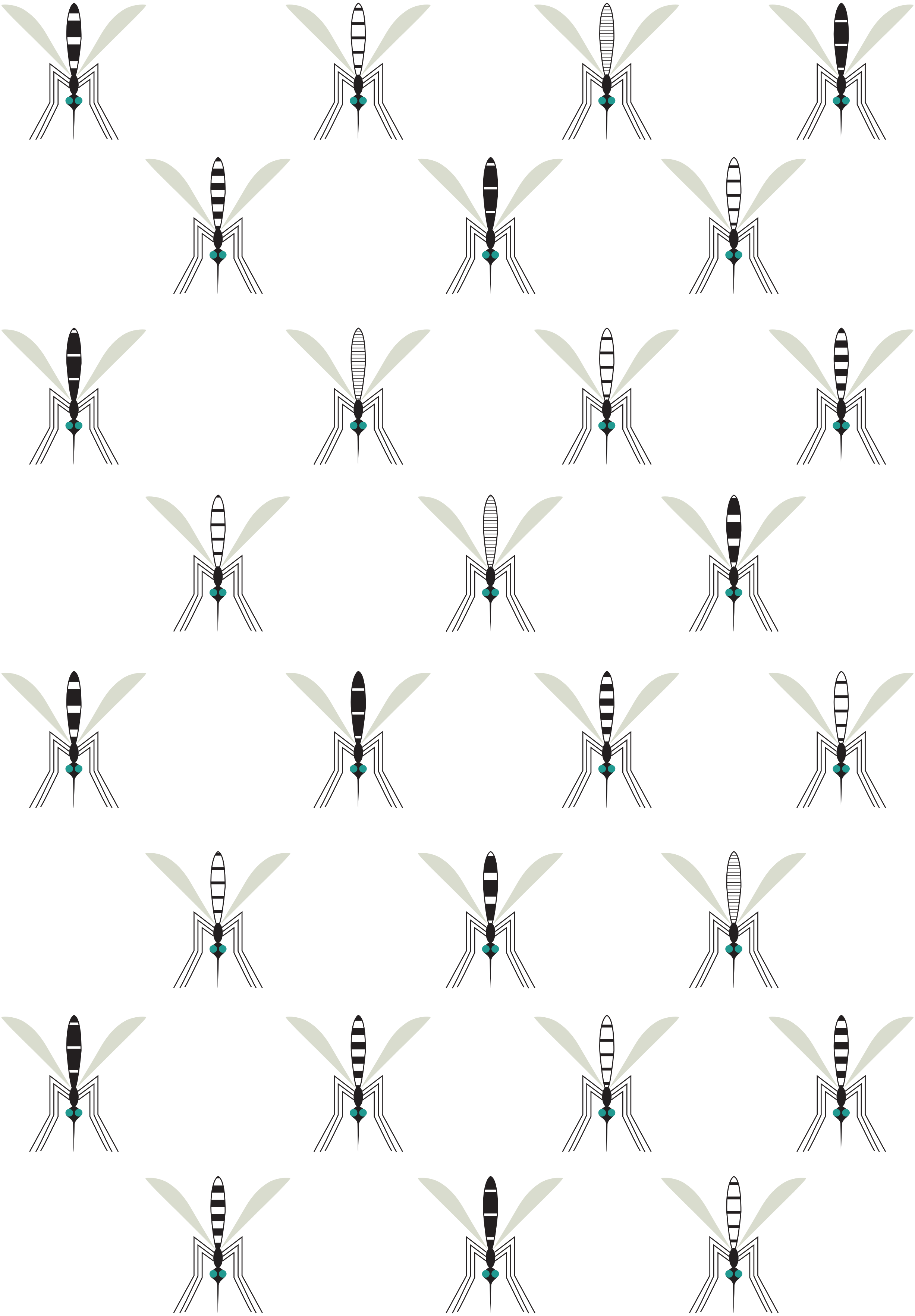

Chapter 2

Community analysis of the abundance and diversity of mosquito species (Diptera: Culicidae) in three European countries at different latitudes

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Abstract

Background: Studies on mosquito species diversity in Europe often focus on a specific habitat, region or country. Moreover, different trap types are used for these sampling studies, making it difficult to compare and validate results across Europe. To facilitate comparisons of trapping sites and community analysis, the present study used two trap types for monitoring mosquito species diversity in three habitat types for three different countries in Europe.

Methods: Mosquitoes were trapped using Biogents Sentinel (BGS), and Mosquito Magnet Liberty Plus (MMLP) traps at a total of 27 locations in Sweden, The Netherlands and Italy, comprising farm, peri-urban and wetland habitats. From July 2014 to June 2015 all locations were sampled monthly, except for the winter months. Indices of species richness, evenness and diversity were calculated, and community analyses were carried out with non-metric multidimensional scaling (NMDS) techniques.

Results: A total of 11,745 female mosquitoes were trapped during 887 collections. More than 90% of the mosquitoes belonged to the genera *Culex* and *Aedes*, with *Culex pipiens* being the most abundant species. The highest mosquito diversity was found in Sweden. Within Sweden, species diversity was highest in wetland habitats, whereas in The Netherlands and Italy this was highest at farms. The NMDS analyses showed clear differences in mosquito communities among countries, but not among habitat types. The MMLP trapped a higher diversity of mosquito species than the BGS traps. Also, MMLP traps trapped higher numbers of mosquitoes, except for the genera *Culex* and *Culiseta* in Italy.

Conclusions: A core mosquito community could be identified for the three countries, with *Culex pipiens* as the most abundant species. Differences in mosquito species communities were more defined by the three countries included in the study than by the three habitat types. Differences in mosquito community composition across countries may have implications for disease emergence and further spread throughout Europe. Future research should, therefore, focus on how field data of vector communities can be incorporated into models, to better assess the risk of mosquito-borne disease outbreaks.

Keywords: Disease vectors, community composition, nonmetric multidimensional scaling, host-seeking behaviour, vector surveillance

Introduction

Intensified movement of humans, animals, and goods on a global scale in combination with climate change creates opportunities for invasive and often exotic Culicidae vector species to establish in Europe (Schaffner *et al.*, 2009). Even without the arrival of exotic mosquitoes, suitable vector species are already present and may facilitate the successful spread of pathogens (Han *et al.*, 1999; Hubálek & Halouzka, 1999; Higgs *et al.*, 2004; Balenghien *et al.*, 2006; Chevalier *et al.*, 2010; Vogels *et al.*, 2015). The introduction of West Nile Virus (WNV) in the USA is probably the most striking example of a pathogen that was rapidly spread by the local vector community throughout the entire country (Snapinn *et al.*, 2007). Moreover, outbreaks of WNV caused by mosquito vectors in Romania (1996) and Russia (1999) resulted in hundreds of human cases, although rapid spread throughout Europe was not observed (Sambri *et al.*, 2013).

Mild winters in combination with humid and hot summers allow vector populations to proliferate rapidly, resulting in increased mosquito nuisance and vectorial capacity (Semenza & Menne, 2009). Human cases of WNV in Europe were reported during 2016 for Spain, Italy, Austria, Romania, Hungary, Serbia and Ukraine (ECDC, 2016). The continued emergence of arboviruses in southern, eastern, and central Europe justifies the demand for detailed knowledge about the vectors that could transmit pathogens (Hubálek & Halouzka, 1999; Calistri *et al.*, 2010; Sambri *et al.*, 2013). For example, a theoretical modelling study by Roche *et al.* (2013) suggested that higher vector species richness can increase pathogen transmission. In contrast, a study by Chaves *et al.* (2011) suggested that higher diversity in vector communities decreases the risk of amplification and spread of disease. To better understand the role of vector communities in disease spread, knowledge about vector species distribution, abundance, and richness is therefore essential.

In Europe, several mosquito species, including the *Culex pipiens* complex, *Cx. modestus* (Ficalbi, 1889), the *Anopheles maculipennis* complex, *Aedes vexans* (Meigen, 1830) and *Ae. albopictus* (Skuse, 1895), can act as vectors of parasites or viruses like malaria, Zika virus, West Nile virus, or Rift Valley fever virus (Han *et al.*, 1999; Balenghien *et al.*, 2006; Chevalier *et al.*, 2010; Aliota *et al.*, 2016). Thus far, ecological studies on vector species diversity often focused on one specific country (Lundström *et al.*, 2013; Versteirt *et al.*, 2013; Ibanez-Justicia *et al.*, 2015), region within a country (Balenghien *et al.*, 2006; Osório *et al.*, 2008), or even on a single habitat (Schäfer & Lundström, 2001; Schäfer *et al.*, 2004; Rettich *et al.*, 2007; Chaves *et al.*, 2011; Roiz *et al.*, 2015). In addition, mosquito species diversity has mostly been studied with one, rather than with a selection of different surveillance trap types. The use of different trap types in each study makes it difficult to make direct comparisons between them. Given the lack of standardized, cross-European studies, this study aimed to sample and assess mosquito species diversity simultaneously. This was done by using two mosquito trap types, in three representative countries at different latitudes across Europe, and for three different habitat types. With this setup, the differences in species richness, diversity, and community composition in different habitats across different countries in Europe could be identified. In addition, the relative efficiency of two trap types could be compared.

Materials and Methods

Mosquito sampling

Adult mosquitoes were sampled with two trap types: the Biogents Sentinel (BGS) trap (BioGents GmbH, Germany, http://www.biogents.com/) and the Mosquito Magnet Liberty Plus (MMLP) trap (Woodstream Corp., USA, http://www.mosquitomagnet.com/). For the production of CO_2 in the BGS trap, a mixture of 17.5 g dry instant yeast (Bruggeman, The Netherlands), 250 g white granulated sugar and 2 l of tap water in a 5 l plastic bottle was used (Smallegange *et al.*, 2010). For the MMLP trap, combustion of propane provided CO_2.

Sampling locations

The traps were placed in three countries at different latitudes across Europe: southern Sweden (surroundings of Linköping 58.410808N, 15.621532E, 45 m elevation), the central part of The Netherlands (surroundings of Wageningen 51.964795N, 5.662898E, 9 m elevation), and central Italy (surroundings of San Benedetto del Tronto 42.949483N, 13.878503E, 4 m elevation). In each country, three habitat types were sampled: (i) wetlands, (ii) farms, and (iii) peri-urban areas (Fig. 1). Wetlands are often considered as primary spots for transmission of vector-borne diseases as both reservoirs (birds), susceptible hosts (large grazers), and vectors (mosquitoes) can be present at a single location (Ezenwa *et al.*, 2007; Reusken *et al.*, 2010; Roiz *et al.*, 2015). Farms were sampled because vector-borne diseases can have a large impact on livestock welfare, associated with high economic loss. Peri-urban areas are hypothesized to have a higher likelihood of human infection with a zoonosis, because of their location at the periphery of urban areas and proximity to farmland areas (Maassen *et al.*, 2012). Each habitat type was represented by three unique sampling locations (Fig. 1), each separated by at least 100 m. At these locations, traps were placed at a minimum distance of 1 m from any walls or fences and were sheltered from the wind, rainfall, and direct sunlight as much as possible.

Trap locations in wetlands had a minimum of 50% marshy or standing water within a 100 m radius of the traps. The farms selected for sampling had at least 100 dairy cows, except for locations 10, 20 and 21 (Vogels *et al.*, 2016), which had a minimum of ten dairy cows. Traps were placed within 50 m of an open livestock stable present at the farm. Peri-urban locations were at the periphery of a city (inhabitants < 150,000). Within a 50 m radius of the trap, at least two occupied residential properties were present. Gardens were open, except for two locations (13 and 14 in The Netherlands) that were bordered on least at three sides of the garden with fences of 2 m height.

Habitat types matched the classification of the CORINE European Land cover database (EEA, 2000), although habitats classified as wetlands for the present study were on some occasions classified as ‘agricultural’ or ‘natural forest area’. One of the peri-urban sites in Italy was

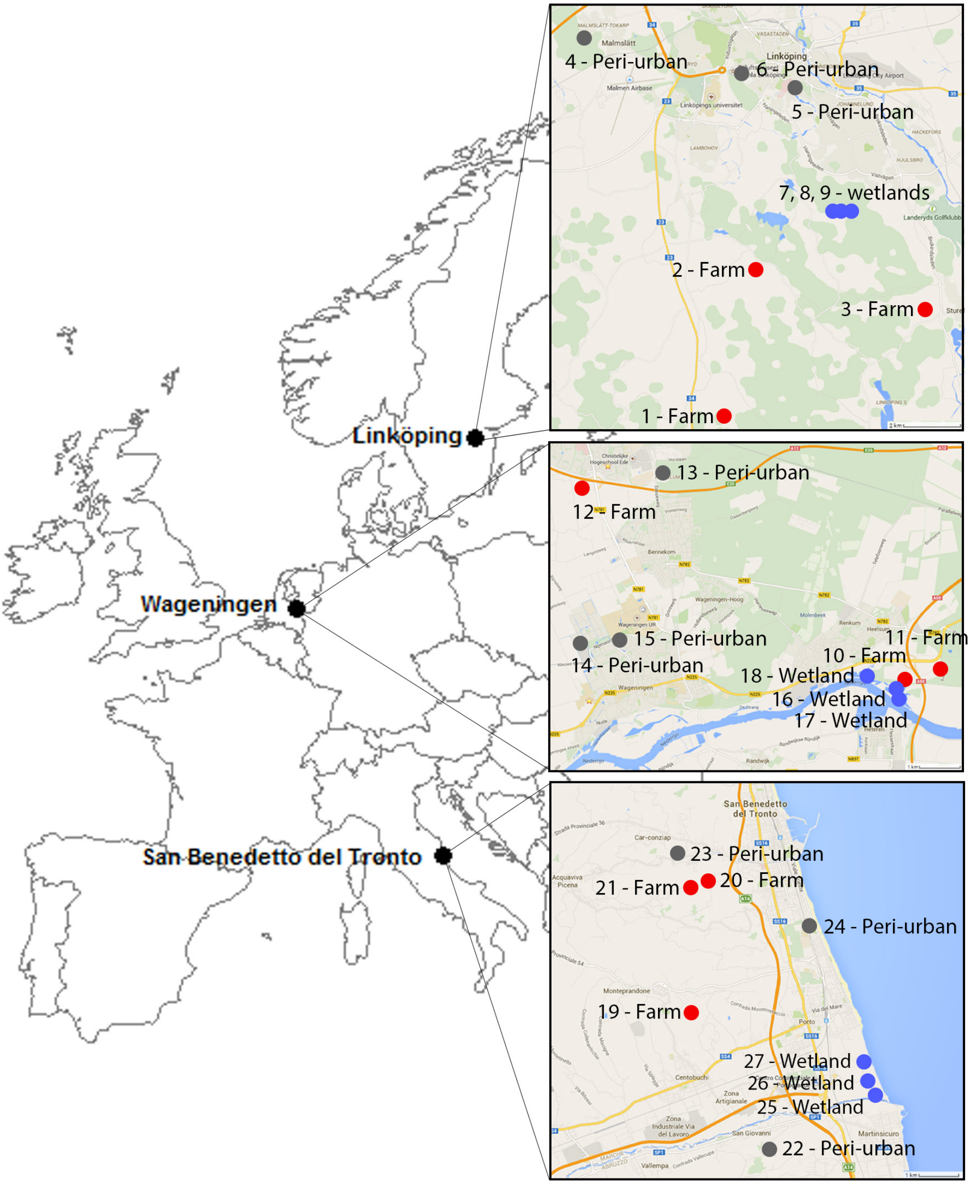
4 - Peri-urban
6 - Peri-urban
5 - Peri-urban
7, 8, 9 - wetlands
2 - Farm
3 - Farm
1 - Farm
Linköping
13 - Peri-urban
12 - Farm
Wageningen
15 - Peri-urban
14 - Peri-urban
11 - Farm
10 - Farm
18 - Wetland
16 - Wetland
17 - Wetland
San Benedetto del Tronto
23 - Peri-urban
21 - Farm
20 - Farm
24 - Peri-urban
19 - Farm
27 - Wetland
26 - Wetland
25 - Wetland
22 - Peri-urban

Figure 1. Overview of selected study sites. Overview of selected sites (1 to 27, see Vogels et al. (2016) for more details about the locations) within each of the three countries in Europe: Linköping, Sweden (58.410808N, 15.621532E); Wageningen, The Netherlands (51.964795N, 5.662898E); San Benedetto del Tronto, Italy (42.949483N, 13.878503E). Farm habitats are indicated with a red dot (1–3, 10–12, 19–21), peri-urban habitats with a grey dot (4–6, 13–15, 22–24), and wetland habitats with a blue dot (7–9, 16–18, 25–27).

classified as ‘agricultural area’ instead of ‘artificial surfaces’ within the CORINE database, most likely because it was situated at the very edge of the city.

Sampling procedures

Collections were performed monthly during six consecutive days in each country. Within each month, the exact timing of the sampling period varied for the three countries. Traps were active for 24 h and were emptied and rotated among the sampling locations (three trapping locations, in three different habitat types, and three countries) between sunrise and sunset of the next day. Sampling took place from July 2014 to June 2015, except for the winter months December, January and February (and March for Sweden). Mosquitoes were stored at -20 °C in Eppendorf tubes containing small silica beads covered with cotton wool.

Sample identification

All female mosquitoes were identified to species level using the key of Becker *et al.* (2010). Morphologically similar species were recorded as belonging to a complex of one of the following species: *An. claviger*, *An. maculipennis*, *Ae. cantans*, *Ae. caspius*, *Ae. cinereus*, *Ae. detritus* and *Cx. pipiens*. These names are used throughout the remainder of the manuscript as a representative for all species in each complex. For the taxonomy of *Aedini* species, the classification of Becker *et al.* (2010) and Wilkerson *et al.* (2015) was used.

Statistical analyses

Species diversity and evenness were calculated for the three countries and the farm, peri-urban, and wetland habitats. In addition, diversity indices were calculated for the two trap types (BGS and MMLP). Simpson’s Index of Diversity was calculated; $1 - D = 1 - \frac{\sum n_i(n_i-1)}{N(N-1)}$, where n_i is the number of the i^{th} species and N is the total number of specimens in the studied country or habitat. Simpson’s Index of Diversity reflects the probability that two individuals taken at random from the dataset are not the same species. Values for Simpson’s Index of Diversity range between 0 and 1, with larger values representing greater diversity. The Shannon-Wiener’s Diversity Index was also used as a diversity index and calculated as $H' = -\sum_{i=1}^{R} p_i \ln(p_i)$, where $p_i = \frac{n_i}{N}$. The Shannon-Wiener’s Diversity Index is based on the uncertainty that an individual taken at random from the dataset is predicted correctly as a certain species. Larger values represent larger uncertainty, thus greater diversity. This method is sensitive to sample size, whereas the Simpson’s Index puts more weight on dominant species and is hardly influenced by a few rare species. In addition, the Shannon-Wiener’s evenness was calculated as $E = \frac{H'}{\ln(S)}$, where S is the total number of species for the country or habitat. Values range between 0 and 1, where 1 is complete evenness, i.e. all species being equally abundant.

The effect of trap type on the number of mosquitoes per genera was analysed using a Mann-Whitney-Wilcoxon test, as these data were not normally distributed and variance was unequal. To better understand whether sufficient trapping efforts have been made for a reasonable estimate of species diversity, a rarefaction curve of the species and the number of collected mosquitoes were created with the rarecurve function within the VEGAN version 2.9.2. package (Oksanen *et al.*, 2009) in R version 3.2.3 (R Development Core Team, 2015).

To examine the combined effect of country, habitat, and diversity on the mosquito community composition, nonmetric multidimensional scaling (NMDS) analyses were performed. This method of data analysis creates a spatial ordination based on proximities between the elements of interest (habitat type, country, mosquito species, and mosquito abundance in this case) (Young *et al.*, 1995). The degree of stress for each NMDS plot was calculated, which indicates the reliability of the outcome, i.e. lower stress corresponds with a higher reliability. The ordination of elements is considered arbitrary with stress-values of 0.3 or above. The dissimilarity matrices are based on abundances for each species within the community. Distances between points were determined with the metaMDS function using the Bray-Curtis dissimilarity metric. All data were analysed in the statistical software package R version 3.2.3 (R Development Core Team, 2015).

Results

A total of 887 trap collections were performed in Sweden, The Netherlands and Italy. In 617 (70%) of these collections, one or more mosquitoes were trapped. The BGS trap and MMLP trap ran effectively on 457 and 430 occasions, respectively.

A total of 11,745 mosquitoes were trapped during this study. Of these, 10,191 (87%) female mosquitoes could be identified to species level. Other individuals were either males (1376; 11.7%) or damaged (178; 1.5%) to the extent that they could not be identified morphologically. Over the three countries, a total of 40 mosquito species were found, comprising six genera. The rarefaction plots for each of the three countries are beyond their exponential growth curve, and level off (Additional file 1: Figure S1). This shows that our sampling effort was sufficient for obtaining a representative number of species for our locations in the three countries. The total number of female mosquitoes trapped during the field study in the three countries combined was highest for the genus *Culex* (61.6%), followed by *Aedes* (29.4%), *Culiseta* (4.7%), *Anopheles* (3.2%), *Coquillettidia* (1.0%) and *Uranotaenia* (0.2%). The most abundant species was *Culex pipiens* with a total of 5202 (51%) out of all identified female mosquitoes ($n = 10{,}191$) from the three countries.

Overall, the MMLP trapped the largest numbers of mosquitoes in Sweden and Italy, while the BGS trapped most mosquitoes in The Netherlands. In all countries, the MMLP trapped most species and had the highest diversity in the collections trapped (Table 1). Of all 40 mosquito species trapped, 95% were found in the MMLP traps, whereas only 55% were found in the BGS traps.

Table 1. Species diversity indices by trap type. Values for Simpson's Index of Diversity, Shannon-Wiener's diversity and Shannon-Wiener's evenness for two trap types in three countries, Sweden, The Netherlands and Italy.

Taxonomic diversity	Sweden		The Netherlands		Italy	
	BGS	MMLP	BGS	MMLP	BGS	MMLP
No. of samples (trapping nights)	138	136	159	153	160	141
No. of species trapped	14	29	8	12	16	24
No. of specimens trapped	270	1028	2397	899	2108	3489
Simpson's Diversity Index	0.776	0.877	0.091	0.475	0.469	0.722
Shannon-Wiener's diversity	1.874	2.377	0.225	0.942	1.033	1.611
Shannon-Wiener's evenness	0.71	0.706	0.108	0.379	0.373	0.507

Abbreviations: BGS, Biogents Sentinel trap; MMLP, Mosquito Magnet Liberty Plus trap

As the study design was the same for all habitats and countries, we can compare mosquito abundances between the two trap types. From the comparisons between the two traps, the MMLP collected significantly more mosquitoes per 24 hours in six out of twelve comparisons: *Aedes* mosquitoes in Sweden and Italy, *Anopheles* mosquitoes in Sweden, The Netherlands and Italy, and *Culiseta* mosquitoes in The Netherlands. The BGS trapped significantly more for two out of twelve comparisons: *Culex* and *Culiseta* mosquitoes in Italy (Additional file 2: Figure S2). In the remaining four comparisons, both traps collected equal numbers of mosquitoes.

Although the number of samples taken, and specimens trapped in Sweden was the lowest, the highest species diversity, richness, and evenness were found here compared to the other two countries. The lowest values for diversity were found in The Netherlands (Table 2). The species richness and diversity of the habitats differed among countries. In Sweden, most species were trapped in the peri-urban habitat, while most species were trapped at farms in The Netherlands, and wetlands in Italy. Farms had the lowest species richness both in Italy and Sweden, while peri-urban habitats had the lowest species richness in The Netherlands (Table 2). Species diversity was highest in Swedish wetlands, whereas it was highest at farms within The Netherlands. In Italy diversity was comparable among habitats (Table 2).

Table 2. Mosquito species diversity by country and habitat. Estimators of taxonomic diversity with values for Simpson's Index of Diversity, Shannon-Wiener's diversity and Shannon-Wiener's evenness for three habitats (farms, peri-urban and wetlands) in three countries (Sweden, The Netherlands, and Italy).

Species	Sweden				The Netherlands				Italy				Total
	Farms	Peri-urban	Wetlands	Total	Farms	Peri-urban	Wetlands	Total	Farms	Peri-urban	Wetlands	Total	
No. of specimens trapped	258	213	827	1298	591	1541	1164	3296	265	501	4831	5597	10191
No. samples	91	91	92	274	99	105	108	312	98	101	102	301	887
No. species trapped	13	24	19	29	11	5	10	14	13	14	21	26	40
Simpson's Index of Diversity	0.739	0.753	0.849	0.885	0.524	0.035	0.153	0.217	0.646	0.611	0.706	0.737	0.699
Shannon-Wiener's diversity	1.667	2.062	2.153	2.425	0.857	0.096	0.395	0.482	1.502	1.259	1.439	1.62	1.803
Shannon-Wiener's evenness	0.65	0.649	0.731	0.72	0.358	0.06	0.171	0.183	0.586	0.477	0.473	0.497	0.489

Table 3. Mosquito species abundance by country and habitat. List of mosquito species with number of specimens for each country (Sweden, The Netherlands, and Italy) and habitat type (farms, peri-urban and wetlands).

Species	Sweden				The Netherlands				Italy				Total
	Farms	Peri-urban	Wetlands	Total	Farms	Peri-urban	Wetlands	Total	Farms	Peri-urban	Wetlands	Total	
Aedes albopictus	0	0	0	0	0	0	0	0	37	272	4	313	313
Aedes behningi	0	2	13	15	0	0	0	0	0	0	1	1	16
Aedes berlandi	0	0	0	0	0	0	0	0	0	1	0	1	1
Aedes cantans	0	4	3	7	0	0	0	0	0	0	0	0	7
Aedes caspius	0	1	0	1	0	0	0	0	5	9	1664	1678	1679
Aedes cataphylla	0	8	0	8	0	0	0	0	0	0	0	0	8
Aedes cinereus	0	3	120	123	0	0	50	50	0	0	4	4	177
Aedes communis	0	0	0	0	0	0	2	2	0	0	0	0	2
Aedes detritus	1	7	131	139	1	0	0	1	12	41	282	335	475
Aedes geniculatus	0	1	0	1	0	0	0	0	0	1	2	3	4
Aedes hexodontus	1	0	0	1	0	0	0	0	0	0	0	0	1
Aedes impiger	0	0	0	0	0	0	0	0	0	1	0	1	1
Aedes intrudens	1	1	0	2	0	0	0	0	0	0	4	4	6
Aedes leucomelas	0	0	2	2	0	0	0	0	0	0	0	0	2
Aedes mercurator	0	0	33	33	0	0	0	0	0	0	0	0	33
Aedes pullatus	0	4	228	232	0	0	0	0	0	0	0	0	232
Aedes riparius	0	0	2	2	0	0	0	0	0	0	0	0	2
Aedes rossicus	0	0	1	1	0	0	0	0	0	0	0	0	1
Aedes vexans	1	5	6	12	1	0	13	14	0	0	7	7	33
Anopheles algeriensis	0	1	0	1	0	0	0	0	0	0	0	0	1
Anopheles claviger	35	8	6	49	2	0	1	3	0	1	3	4	56
Anopheles maculipennis	114	2	96	212	3	1	6	10	7	2	3	12	234
Anopheles plumbeus	16	3	0	19	4	0	1	5	0	11	0	11	35
Anopheles sacharovi	0	0	0	0	0	0	0	0	1	0	1	2	2
Coquillettidia richiardii	4	23	76	103	1	0	2	3	0	0	1	1	107
Culex laticintus	0	0	0	0	0	0	0	0	4	0	27	31	31
Culex martinii	0	0	0	0	0	0	0	0	9	4	878	891	891
Culex mimeticus	0	1	6	7	0	0	0	0	0	0	0	0	7
Culex modestus	5	13	2	20	0	1	1	2	0	1	58	59	81
Culex pipiens	48	100	57	205	316	1514	1070	2900	149	148	1800	2097	5202
Culex pusillus	0	0	0	0	1	0	0	1	0	0	16	16	17
Culex theileri	0	2	0	2	0	0	0	0	2	0	41	43	45
Culiseta alaskaensis	1	1	0	2	0	0	0	0	0	0	0	0	2
Culiseta annulata	27	20	16	63	258	24	18	300	3	3	2	8	371
Culiseta bergrothi	4	1	0	5	3	0	0	3	0	0	0	0	8
Culiseta longiarealata	0	0	0	0	0	0	0	0	34	6	17	57	57
Culiseta morsitans	0	1	28	29	0	0	0	0	1	0	0	1	30
Culiseta ochroptera	0	1	1	2	0	0	0	0	0	0	0	0	2
Culiseta subochrea	0	0	0	0	1	1	0	2	1	0	0	1	3
Uranotaenia unguiculata	0	0	0	0	0	0	0	0	0	0	16	16	16

From the 1298 mosquitoes trapped in Sweden, 29 species were identified. Of these mosquitoes, *Ae. pullatus* (Coquillett, 1904) (18%), *An. maculipennis* (16%), *Cx. pipiens* (16%), *Ae. detritus* (11%) and *Coquillettidia richiardii* (Ficalbi, 1889) (8%), were the most common species (Table 3). The 827 mosquitoes trapped from wetlands in Sweden were dominated by *Aedes* species, most notably *Ae. pullatus* (28%), *Ae. detritus* (16%) and *Ae. cinereus* (Meigen 1818) (15%). For the 258 mosquitoes trapped on farms, the dominating species were *An. maculipennis* (44%), *Cx. pipiens* (19%), and *An. claviger* (14%), whereas the 213 mosquitoes trapped in peri-urban habitats were dominated by *Cx. pipiens* (46%), *Cq. richiardii* (11%), and *Culiseta annulata* (Schrank 1776) (9%).

The Netherlands had the lowest species richness, with 14 species identified in the 3296 mosquitoes trapped during the study period. The most common species found were *Cx. pipiens* (88%) and *Cs. annulata* (9%) (Table 3). Both in the wetland (1164 mosquitoes) and peri-urban (1541 mosquitoes) habitats *Cx. pipiens* was the dominating species with 92% and 98% of the trapped mosquitoes, respectively. From the 591 mosquitoes trapped at farms *Cx. pipiens* (53%) and *Cs. annulata* (44%) were trapped in almost equal number.

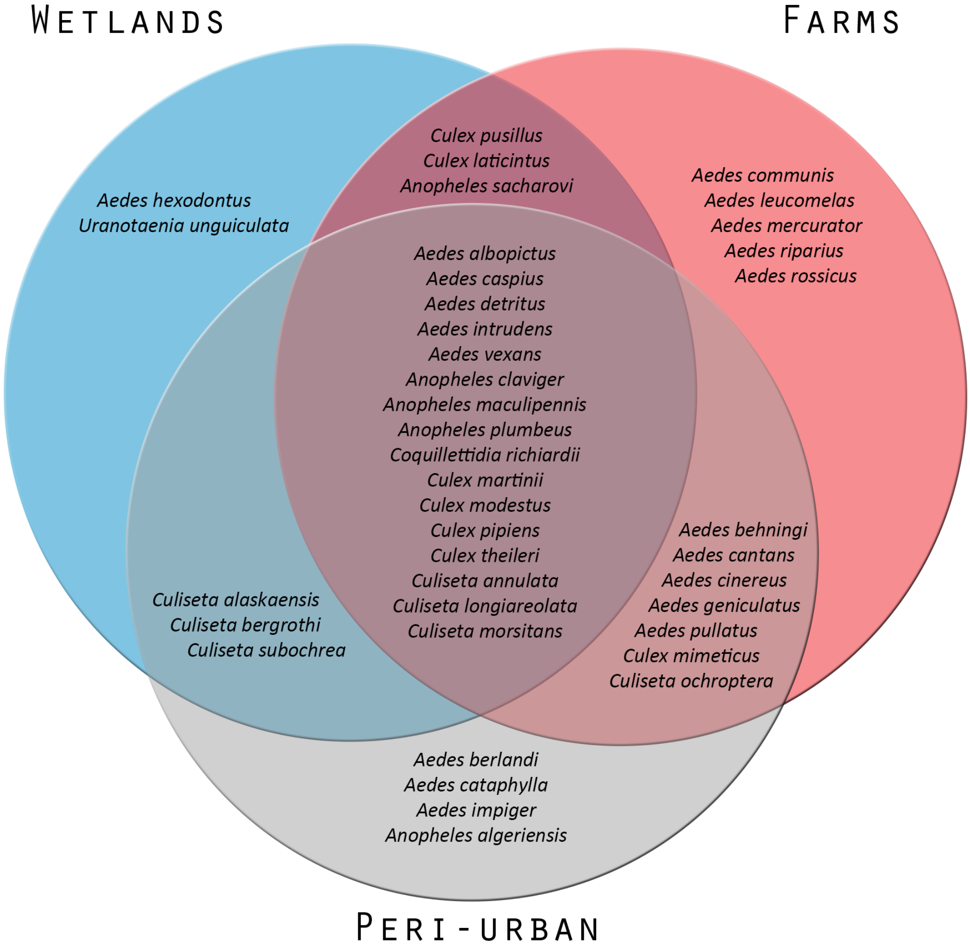
WETLANDS
FARMS
Culex pusillus
Culex laticintus
Anopheles sacharovi
Aedes hexodontus
Uranotaenia unguiculata
Aedes communis
Aedes leucomelas
Aedes mercurator
Aedes riparius
Aedes rossicus
Aedes albopictus
Aedes caspius
Aedes detritus
Aedes intrudens
Aedes vexans
Anopheles claviger
Anopheles maculipennis
Anopheles plumbeus
Coquillettidia richiardii
Culex martinii
Culex modestus
Culex pipiens
Culex theileri
Culiseta annulata
Culiseta longiareolata
Culiseta morsitans
Aedes behningi
Aedes cantans
Aedes cinereus
Aedes geniculatus
Aedes pullatus
Culex mimeticus
Culiseta ochroptera
Culiseta alaskaensis
Culiseta bergrothi
Culiseta subochrea
Aedes berlandi
Aedes cataphylla
Aedes impiger
Anopheles algeriensis
PERI-URBAN

Figure 2. Venn diagram of habitats. Diagram shows the absolute presence of mosquito species found in farm (*red*), peri-urban (*grey*), and wetland (*blue*) habitats.

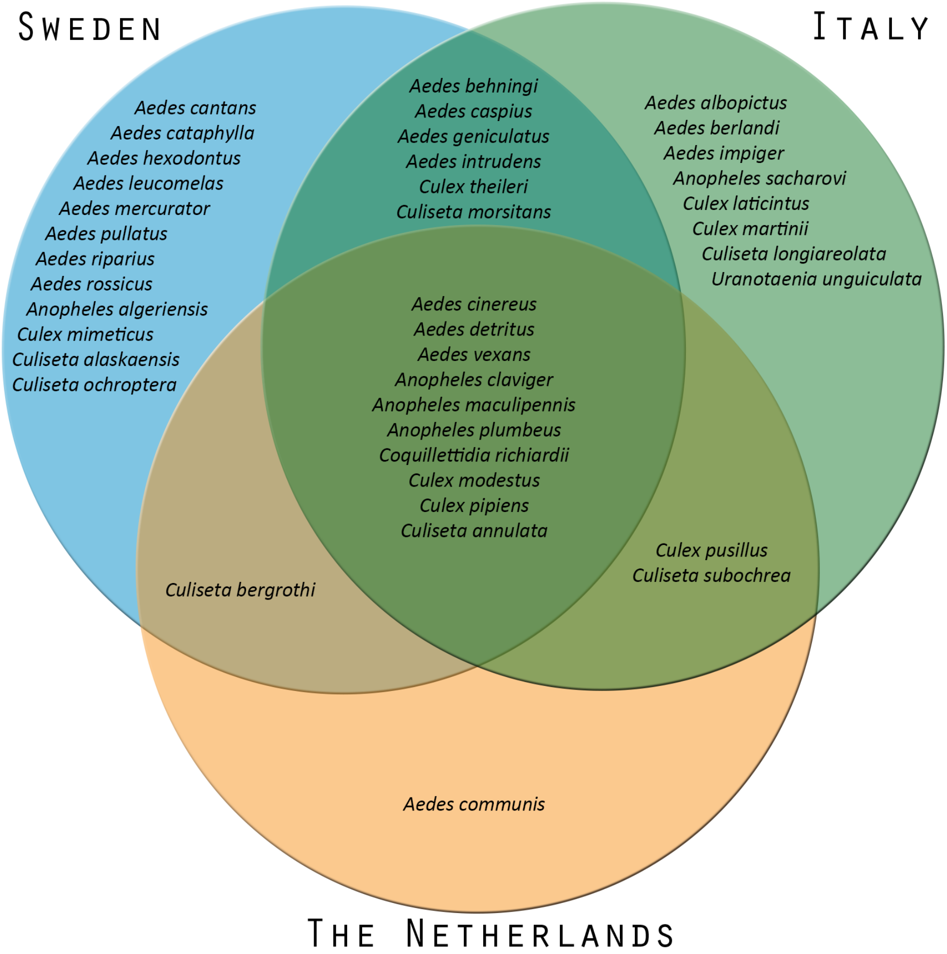
Sweden
Italy
The Netherlands
Aedes cantans
Aedes cataphylla
Aedes hexodontus
Aedes leucomelas
Aedes mercurator
Aedes pullatus
Aedes riparius
Aedes rossicus
Anopheles algeriensis
Culex mimeticus
Culiseta alaskaensis
Culiseta ochroptera
Aedes behningi
Aedes caspius
Aedes geniculatus
Aedes intrudens
Culex theileri
Culiseta morsitans
Aedes albopictus
Aedes berlandi
Aedes impiger
Anopheles sacharovi
Culex laticintus
Culex martinii
Culiseta longiareolata
Uranotaenia unguiculata
Aedes cinereus
Aedes detritus
Aedes vexans
Anopheles claviger
Anopheles maculipennis
Anopheles plumbeus
Coquillettidia richiardii
Culex modestus
Culex pipiens
Culiseta annulata
Culiseta bergrothi
Culex pusillus
Culiseta subochrea
Aedes communis

Figure 3. Venn diagram of countries. Diagram shows the absolute presence of mosquito species found in Sweden (*blue*), The Netherlands (*orange*), and Italy (*green*).

A total of 26 species was identified from the 5597 mosquitoes trapped in Italy, of which *Culex pipiens* (37%), *Ae. caspius* (30%), and *Cx. martinii* (Medschid, 1930) (16%) were the most dominant (Table 3). Wetland habitats (4831 mosquitoes) were mostly populated by these three species. The 265 mosquitoes trapped at Italian farms were dominated by *Cx. pipiens* (56%), *Ae. albopictus* (14%) and *Cs. longiareolata* (Marcquart, 1838) (13%), whereas in peri-urban habitats *Ae. albopictus* was the most abundant species with 54% of the 501 mosquitoes trapped, followed by *Cx. pipiens* (30%) and *Ae. detritus* (8%).

Most mosquito species (29/40, 73%) were found in at least two habitats. Five species occurred exclusively at farms, four species exclusively in peri-urban and two species exclusively in wetland habitats (Fig. 2). All these 11 species were found in one country only, indicating that they are unique trappings (Fig. 3). Furthermore, more than half of the 40 species were trapped in only one of the countries (21/40, 53%), while 25% (10/40) of the species were trapped in all countries (Fig. 3). The latter group included the most abundant species from the three countries: *An. maculipennis*, *Cx. pipiens*, *Ae. detritus*, *Cq. richiardii* and *Cs. annulata*. The most abundant mosquito in Sweden, *Ae. pullatus* only occurred in Swedish farm and wetland habitats. The second and third most abundant species from Italy (*Ae. caspius* and *Cx. martinii*) were trapped in all three habitats, but not in all countries (Figs. 2, 3).

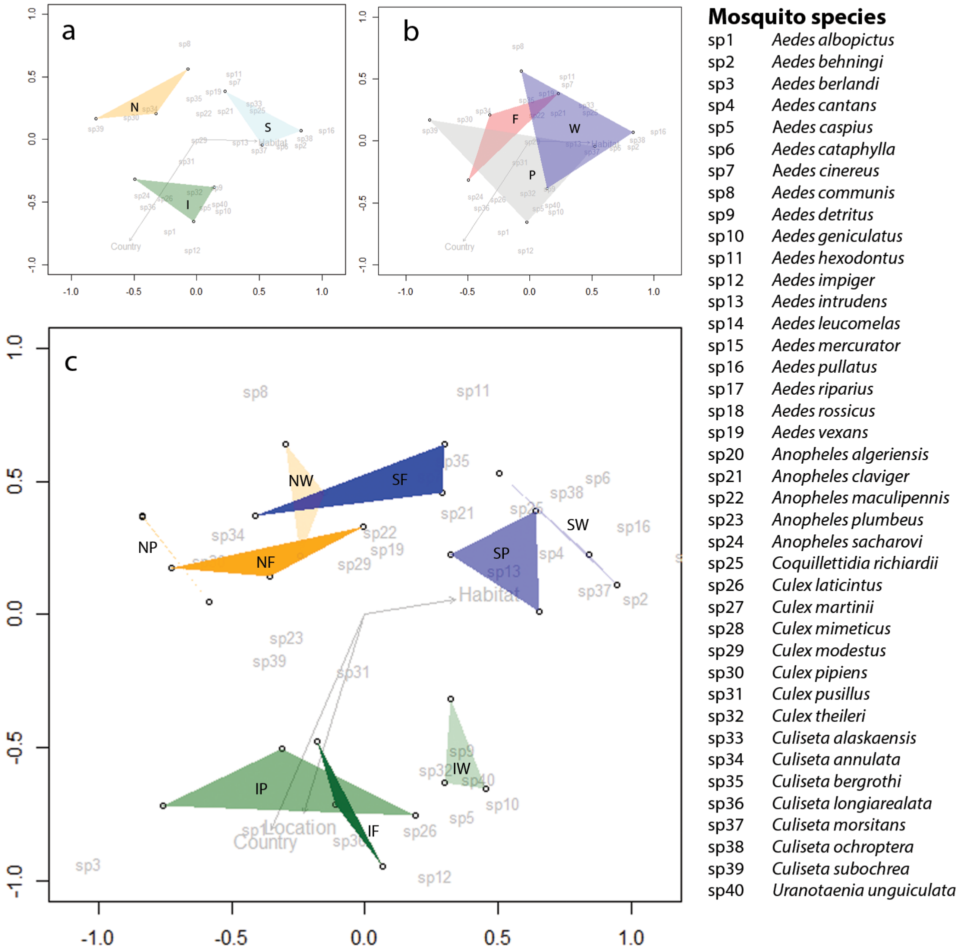

Figure 4. Results of NMDS analyses. a Mosquito community compositions for the three countries. *Abbreviations*: S, Sweden; N, The Netherlands; I, Italy. **b** NMDS analysis for the farm (F), peri-urban (P) and, wetland (W) habitats based on the number of mosquitoes trapped per species in each habitat and country. **c** NMDS analysis based on the number of mosquitoes trapped per species at each location in each country and habitat (Sweden in *blue*: SF, SP, and SW, The Netherlands in *orange*: NF, NP, and NW, Italy in *green*: IF, IP, and IW). The Bray-Curtis dissimilarity index was used for determination of dissimilarities among mosquito community compositions. Stress-value=0.119 for panels A and B, which indicates a good fit of the model. Stress-value=0.197 for panel C, which indicates a reasonable fit of the model.

Dissimilarity matrices resulting from NMDS analyses reveal clear differences in mosquito community composition among countries (stress-value=0.119, *p*=0.029) (Fig. 4a). No significant habitat differences among communities were found (stress-value=0.119, *p*=0.537) (Fig. 4b). However, differences in mosquito communities among habitats within each country were found for some of the habitats (Fig. 4c). Habitat communities differed from each other in Sweden (stress-value=0.121, *p*=0.03) and Italy (stress-value=0.088, *p*=0.033), but were not significantly different from each other in The Netherlands (stress-value=0.041, *p*=0.173).

Discussion

To assess mosquito community diversity at a European scale, the present study used a standardized trapping protocol to sample mosquitoes in three countries at different latitudes across Europe. The highest mosquito diversity was found when trapping with the MMLP trap compared to the BGS trap. Although the BGS was initially developed for trapping host-seeking *Aedes* spp. (Biogents, 2015), in the present study it did not trap large numbers of *Aedes* mosquitoes compared to the MMLP trap. On the other hand, it did trap significantly more *Culex* and *Culiseta* mosquitoes in Italy (Additional file 2: Figure S2). These results differ from the findings of a study in which four trap types in Germany were compared, and where the BGS was the most efficient trap (Luhken *et al.*, 2014). However, that study used different attractive blends for each of their traps, possibly explaining the disparity with our results.

Although the CO_2 used in the present study (*via* propane combustion or sugar fermentation) attracts mosquitoes (Guerenstein & Hildebrand, 2008), more specimens may be trapped if a lure or attractive blend is added to the traps (Roiz *et al.*, 2012; van Loon *et al.*, 2015). However, it is not clear whether there is a selective effect of specific blends on the attraction of different mosquito species. As CO_2 is a general host-seeking cue for blood-feeding arthropods, only CO_2 was chosen as an attractant for this study. Larval sampling could further complement adult female trapping to study mosquito diversity (Rueda, 2008; Lundström *et al.*, 2013; Schaffner & Mathis, 2013). Furthermore, the number of trapped species and specimens can fluctuate substantially depending on the year (Osório *et al.*, 2008). Our data were collected for one year only, and do therefore not take into account annual variation in mosquito population dynamics.

Mosquito community composition differed among countries. This is illustrated by the diversity indices calculated (Table 2), which was highest in Sweden, followed by Italy, and The Netherlands. Also, the Venn-diagram (Fig. 3) shows that 25% of the trapped mosquito species were found in all three countries. Finally, the dissimilarity matrix (Fig. 4a) distinguishes different mosquito communities among countries. However, a core community seemed to be present in all countries (Fig. 3). This core community includes the five most abundant species from the three countries: *An. maculipennis*, *Cx. pipiens*, *Ae. detritus*, *Cq. richiardii* and *Cs. annulata*. Although this core community occurs throughout the sampled countries, it cannot be assumed that their contribution to disease spread is similar in all countries. Species or biotypes within the *An. maculipennis* or *Cx. pipiens* complexes can, for example, differ in their feeding behaviour or vector competence (Becker *et al.*, 2010; Gomes *et al.*, 2013; Vogels *et al.*, 2016) and thus play different roles in pathogen transmission.

In total, 29 of 49 mosquito species officially recorded for Sweden (Lundström *et al.*, 2013), 14 of 35 species for The Netherlands (Ibanez-Justicia *et al.*, 2015), and 22 of 64 species for Italy (Severini *et al.*, 2009) were trapped during this study. Although our sampling effort was comprehensive, as can be seen in the rarefaction plot (Additional file 1: Figure S1), it should be mentioned that mosquito diversity in our collections is not representative for the countries as a whole. Results can be compared among the three countries in this study because of the consistent study design. However, sampling was done in a small representative area that cannot be extrapolated to the country level. Complete mosquito diversity for a country is better estimated with studies sampling throughout a country with many traps for a longer period, as can be illustrated by the fact that diversity indices found by Ibanez-Justicia *et al.* (2015) are higher for The Netherlands than those in the current study (Table 2). Combining both setups for multiple countries and multiple years would be the ideal study design, but this is not logistically feasible. Mosquito species that were not trapped in this study most likely occur in very low densities or use different habitats than sampled in this study, thereby making them less relevant from the perspective of disease spread.

While community composition differed among countries, they overlapped among habitat types (Fig. 4b). However, when differentiating habitats within countries, there was marked habitat effects on community composition (Fig. 4c). Communities among habitats differed within Sweden and Italy, while communities in The Netherlands were more similar to each other for all habitats. This might be explained by the relatively high level of habitat fragmentation in The Netherlands (Ibanez-Justicia *et al.*, 2015). As a result of high habitat diversity in the landscape on a small spatial scale, species may be more easily collected from nearby habitats.

Although diversity indices did not show a clear pattern for habitats (Table 2), species diversity was always higher in (semi-) natural areas (farms and wetlands) when compared to peri-urban habitats in all countries. This corresponds with other studies that found higher diversity in wet, inundated or heterogenic natural areas with a high vegetation index (Schäfer *et al.*, 2004; Foley *et al.*, 2007; Chaves *et al.*, 2011; Marí & Jiménez-Peydró, 2011; Schaffner & Mathis, 2013; Versteirt *et al.*, 2013; Roiz *et al.*, 2015). This probably reflects the fact that natural areas offer more diversity in breeding habitats, resting places, and available hosts for mosquitoes.

Although the fewest specimens were trapped in Sweden, the highest diversity was recorded here. It is accepted that species diversity in general, and also for mosquitoes, declines towards the pole regions (Rosenzweig, 1995; Schäfer & Lundström, 2001; Foley *et al.*, 2007). However, if natural areas do indeed accommodate more mosquito diversity, this could explain the higher species diversity in Sweden. Also, high species richness in Sweden could be caused by the relatively high number of *Aedes* species trapped, as the tribe Aedini consists of more mosquito species than any of the other tribes in the Palaearctic (Rueda, 2008).

An earlier study in Italian wetlands found 22 species, of which 14 overlapped with what we found in our Italian wetland site. However, their samples from the same location (Sentina wetlands, 42.901956N, 13.905395E) only comprised six out of the 21 species trapped within the present study (Toma *et al.*, 2008). The greater number of species trapped during our study compared to collections by Toma *et al.* (2008), may be the result of the use of different trap types, and the further development and succession within the Sentina wetland natural area that was restored in 2004, as natural wetlands harbour more mosquito species than constructed wetlands (Schäfer *et al.*, 2004).

From the European core mosquito community, several can be identified as (potential) vectors of pathogens. Species from the genera *Culex* and *Aedes* are known to transmit pathogens (Becker *et al.*, 2010). *Culex pipiens* was trapped in large numbers in all three countries and most of the habitats. Other studies in Europe also found *Cx. pipiens* to be one of the most dominant species (Aranda *et al.*, 2009; Marí & Jiménez-Peydró, 2011; Schaffner & Mathis, 2013; Versteirt *et al.*, 2013; Roiz *et al.*, 2015; Boukraa *et al.*, 2016). *Culex pipiens* is a known vector for WNV, which already circulates in some parts of Europe (Han *et al.*, 1999; Turell *et al.*, 2005). It is still unclear why WNV does not spread to more northern countries in Europe (Hubálek & Halouzka, 1999; Calistri *et al.*, 2010; Sambri *et al.*, 2013), but the temperature seems to be one of the main driving factors (Fros *et al.*, 2015; Vogels *et al.*, 2016).

Aedes albopictus is a known vector of approximately 22 arboviruses, including WNV, dengue, chikungunya, and possibly Zika (Gratz, 2004; Petrić *et al.*, 2014; Aliota *et al.*, 2016; Chouin-Carneiro *et al.*, 2016). In our study, they were mainly trapped from peri-urban sites in Italy where they even outnumbered *Cx. pipiens* (Table 3), but they were not found in Sweden or The Netherlands. However, *Ae. albopictus* is known to be repeatedly introduced into The Netherlands with the import of tires and lucky bamboo plants (Reiter, 1998; Scholte *et al.*, 2007). It is expected that *Ae. albopictus* is unable to survive in Sweden, but that it can establish in The Netherlands (Benedict *et al.*, 2007). The introduction and establishment of an efficient vector such as *Ae. albopictus* will significantly increase the risk of pathogen transmission, as was shown in Italy for outbreaks of chikungunya (Rezza *et al.*, 2007). This stresses the need for appropriate monitoring and control strategies against this species.

Other *Aedes* species, such as *Ae. caspius*, *Ae. pullatus*, *Ae. detritus* and *Ae. cinereus*, were mainly trapped in Italian and Swedish wetlands. *Aedes caspius* is considered a potential vector for WNV and tularemia (Becker *et al.*, 2010). The high numbers of *Ae. caspius* mosquitoes trapped in Italian wetlands correspond to its association with brackish water in coastal wetlands (Osório *et al.*, 2008; Aranda *et al.*, 2009; Marí & Jiménez-Peydró, 2011; Roiz *et al.*, 2015). The *Ae. cinereus* mosquitoes, mainly trapped in Swedish wetlands, are considered an important bridge vector for both tularemia bacteria, and the Sindbis virus that is re-emerging in humans every seventh year in northern-European countries (Kurkela, 2008; Lundström *et* *al.*, 2013). Mosquitoes from the species responsible for maintaining the enzootic cycle of the Sindbis virus among birds, *Cx. torrentium* were only found in small numbers in our earlier study (Vogels *et al.*, 2016).

Besides the presence of specific vector species in the European mosquito core community, it is also important to take the diversity of communities associated with these dominant vectors into account (Chaves *et al.*, 2011; Roche *et al.*, 2013). Mosquito community composition differed among countries and for some habitats within countries. Chaves *et al.* (2011) suggest that higher diversity in vector communities is expected to decrease the risk of amplification and spread of a vector-borne disease because higher vector species diversity is thought to be correlated with lower mosquito abundance. In contrast, a theoretical study by Roche *et al.* (2013) suggests that greater species richness can amplify disease transmission. Specific vector species could play an important role in these complex community dynamics. Given the fact that many vector-borne diseases require multiple species that together influence the rate of transmission, understanding the ecology of vector networks is becoming increasingly important.

Conclusions

Within our study in three countries across Europe, a core mosquito community could be identified, with *Culex pipiens* as the most abundant species. Differences in mosquito community composition were more defined by countries than habitats, although some habitats do accommodate distinct communities in specific countries. Differences in vector community composition across countries may have implications for disease emergence and further spread throughout Europe. Both the role of these complex communities as well as the role of specific vector species within these communities should be further determined. To better understand patterns of disease emergence and outbreaks, differences in vector communities should, therefore, be incorporated in mathematical and statistical models.

Acknowledgements

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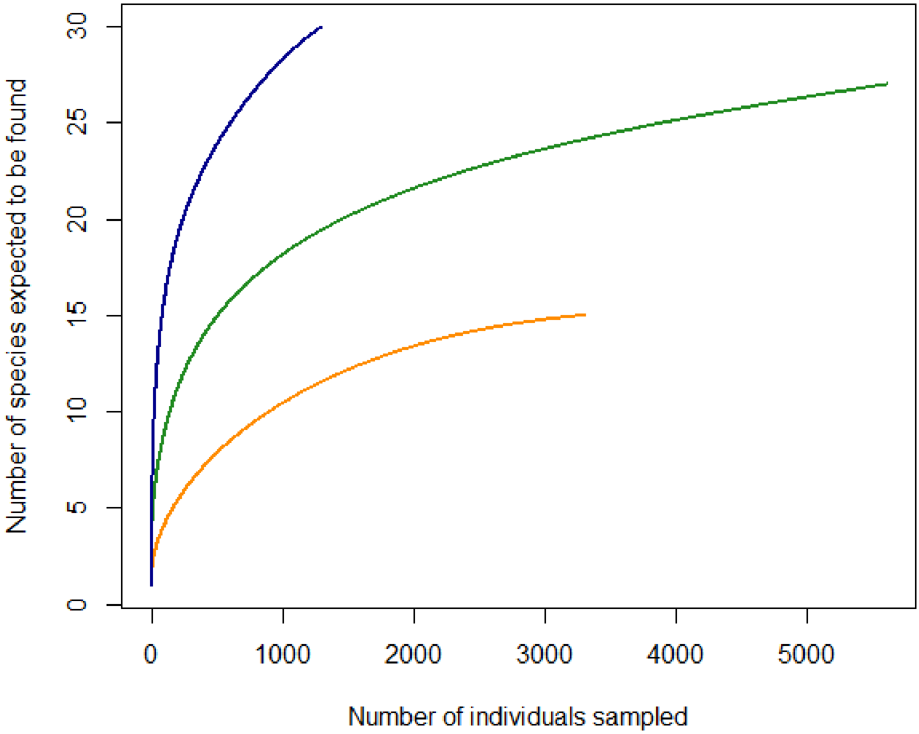
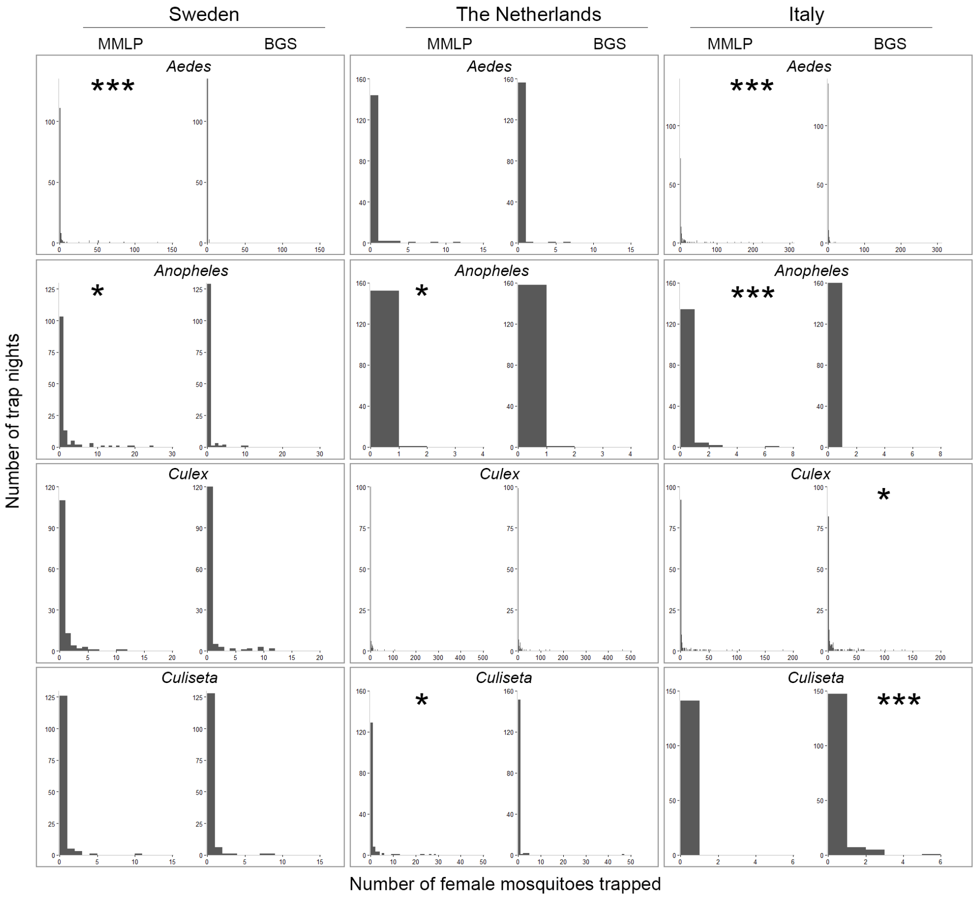
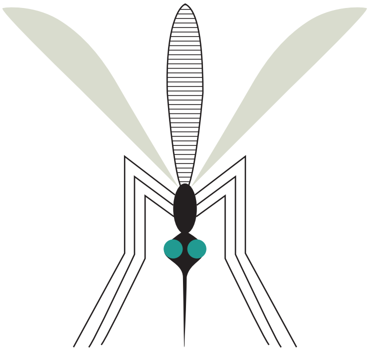
Supplementary



Additional file 1: Figure S1. Rarefaction plot of sampling effort. The plot shows the number of species expected to be found for the number of individuals sampled for Sweden (blue), Italy (green), and The Netherlands (orange).



Additional file 2: Figure S2. Frequency distribution for the number of trap nights that a specific number of female mosquitoes was trapped. Shown are the results for three countries (Sweden, The Netherland, and Italy), two trap types (MMLP, Mosquito Magnet Liberty Plus trap; BGS, Biogents Sentinel trap), and the four most common genera (*Aedes*, *Anopheles*, *Culex* and *Culiseta*). Comparisons between the two trap types were made for the four most common mosquito genera in each country, using the Mann-Whitney-Wilcoxon test. Significance is displayed for each comparison, with $^{*}P<0.05$ and $^{***}P<0.001$.



Chapter 3

Latitudinal diversity of *Culex pipiens* biotypes and hybrids in farm, peri-urban, and wetland habitats in Europe

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Abstract

Despite the presence of *Culex (Cx.) pipiens* mosquitoes and circulation of West Nile virus (WNV), WNV outbreaks have so far not occurred in northern Europe. The species *Cx. pipiens* consists of two morphologically identical biotypes, *pipiens* and *molestus*, which can form hybrids. Until now, population dynamic studies of *Cx. pipiens* have not differentiated between biotypes and hybrids at the European scale, nor have they used comparative surveillance approaches. We therefore aimed to elucidate the relative abundance of *Cx. pipiens* biotypes and hybrids in three habitat types at different latitudes across Europe, using two different surveillance traps. BG-Sentinel and Mosquito-Magnet Liberty Plus traps were placed in three habitat types (farms, peri-urban, wetlands), in three European countries (Sweden, The Netherlands, Italy). Collected *Cx. pipiens* mosquitoes were identified to biotype with real-time PCR. Both trap types collected equal ratios of the biotypes and their hybrids. From northern to southern latitudes there was a significant decrease of *pipiens* and an increase of *molestus*. Habitat types influenced the relative ratios of biotypes and hybrids, but results were not consistent across latitudes. Our results emphasize the need to differentiate *Cx. pipiens* to the biotype level, especially for proper future WNV risk assessments for Europe.

Keywords: West Nile virus; population dynamics; northern house mosquito; biotype *molestus*.

Introduction

Global warming, increased travel and trade, and land-use changes are important drivers for the (re-)emergence of vector-borne diseases, such as West Nile virus (WNV; family: *Flaviviridae*) (Morens *et al.*, 2004). The potential of WNV to quickly spread to new areas is clearly illustrated by the outbreaks that occurred in the United States of America, after the initial introduction in 1999 (Ebel *et al.*, 2004; Davis *et al.*, 2005; Snapinn *et al.*, 2007). WNV outbreaks have also occurred in southern and central European countries, but no outbreaks among humans have occurred in northern Europe (Hubalek & Halouzka, 1999; Calistri *et al.*, 2010; Sambri *et al.*, 2013).

WNV is maintained in an enzootic cycle between birds and mosquitoes. The main vector for WNV is the mosquito *Culex (Cx.) pipiens* (Fonseca *et al.*, 2004; Kilpatrick *et al.*, 2005). The *Cx. pipiens* complex consists of several closely related species and biotypes, of which only the species *Cx. pipiens* (Linnaeus 1758) occurs in Europe (Farajollahi *et al.*, 2011). Because of its similar morphology, the species *Cx. torrentium* (Martini 1925) is often included in taxonomic studies of the *Cx. pipiens* complex (Becker *et al.*, 2012). The species *Cx. pipiens* consists of two morphologically similar biotypes, named *pipiens* (Linnaeus 1758) and *molestus* (Forskål 1775), which show distinct behaviour. Biotype *pipiens* is the most important vector in the enzootic cycle because of its preference for birds (Byrne & Nichols, 1999). During winter, biotype *pipiens* enters diapause, which provides a means of overwintering for WNV (Sanburg & Larsen, 1973; Dohm & Turell, 2001). Biotype *molestus* prefers mammals, including humans, as hosts, and remains active year-round (Marshall & Staley, 1936; Shute, 1941; Willcox, 1941). Host availability can induce a strong shift in host feeding behaviour of biotype *molestus* from mammals to birds, especially in areas with high bird densities (Gomes, Sousa, *et al.*, 2013). Previously, biotype *molestus* has been described as occurring underground (Fonseca *et al.*, 2004), but recent studies show that both biotypes occur sympatrically in aboveground habitats throughout Europe (Rudolf *et al.*, 2013; Osório *et al.*, 2014; Vogels *et al.*, 2015; Di Luca *et al.*, 2016). Furthermore, biotype *pipiens* and biotype *molestus* can form hybrids which show intermediate host preference (Fritz *et al.*, 2015). As a result of this, hybrids can play an important role in bridging WNV from birds to humans (Fonseca *et al.*, 2004).

Several studies elucidated the geographic distribution of the species *Cx. pipiens* and *Cx. torrentium* (Martini 1925) at the European scale (Weitzel *et al.*, 2011; Hesson *et al.*, 2013). In general, *Cx. torrentium* is relatively more abundant in northern Europe, whereas *Cx. pipiens* is more abundant in southern Europe (Hesson *et al.*, 2013). However, these studies did not identify *Cx. pipiens* mosquitoes to the biotype level. Identification to the biotype level is important because the behavioural differences between the two biotypes of *Cx. pipiens* and their hybrids result in different vectorial capacity for WNV. Thus far, in-depth studies that differentiated between the biotypes were done at country level (Gomes, Kioulos, *et al.*, 2013; Rudolf *et al.*, 2013; Osório *et al.*, 2014; Vogels *et al.*, 2015; Di Luca *et al.*, 2016; Zittra *et al.*, 2016). Few of these studies systematically compared biotype ratios among different habitat types (Osório *et al.*, 2014; Di Luca *et al.*, 2016; Zittra *et al.*, 2016). However, due to differences in experimental design it is hard to make direct comparisons between *Cx. pipiens* populations in northern and southern European countries.

The aim of this study was to assess the relative abundance of the *Cx. pipiens* biotypes with two types of traps (Biogents Sentinel and Mosquito Magnet Liberty Plus), in three different habitat types (farms, peri-urban, and wetlands), and in three countries (Sweden, The Netherlands, and Italy) at different latitudes across Europe.

Materials and Methods

Mosquito collections

Adult mosquitoes were collected with the Biogents Sentinel (BGS) trap (BioGents GmbH, Germany) and the Mosquito Magnet Liberty Plus (MMLP) trap (Woodstream Corp., USA). A mixture of 17.5 g dry instant yeast (Bruggeman, The Netherlands), 250 g white granulated sugar and 2 l of tap water in a 5 l plastic bottle was used for CO_2 production in the BGS trap (Smallegange *et al.*, 2010). Combustion of propane provided CO_2 for the MMLP trap.

Both traps were rotated among three trapping locations, in three different habitat types (farms, peri-urban, and wetlands), in Sweden (Linköping), The Netherlands (Wageningen), and Italy (San Benedetto del Tronto; Table 1). The selected farms were dairy cattle farms with a minimum of 10 cows. Traps were placed within 50 m of the open indoor stable. Peri-urban locations were at the periphery of a city (inhabitants <150,000), and within a 50 m radius of the trap, at least two occupied residential properties were present. Locations in a wetland habitat had a minimum of 50% marshy or standing water within a 100 m radius of the traps. Trapping locations were at least 100 m apart.

Collections were done during six consecutive days, every month in each country. Sampling periods were from July 2014 to June 2015, except for the winter months December, January, and February (and March for Sweden). Traps were emptied and repositioned every 24 hours between sunrise and sunset of the next day. Mosquitoes were stored at -20 °C in Eppendorf tubes containing small silica beads covered with cotton wool.

Table 1. Coordinates of all 27 trapping locations in the three different habitat types (farms, peri-urban, and wetlands) in three different European countries (Sweden, The Netherlands, and Italy).

Country	Habitat type	Sampling location	Coordinates
Sweden	Farms	1	58.296530, 15.584782
(Linköping)		2	58.343622, 15.602404
		3	58.330597, 15.704327
	Peri-urban	4	58.416973, 15.499516
		5	58.401515, 15.626744
		6	58.405494, 15.595035
	Wetlands	7	58.362106, 15.651861
		8	58.361585, 15.654910
		9	58.361542, 15.659072
The Netherlands	Farms	10	51.971084, 5.761455
(Wageningen)		11	51.973637, 5.773978
		12	52.013077, 5.645998
	Peri-urban	13	52.018075, 5.655372
		14	51.979257, 5.645230
		15	51.979771, 5.660278
	Wetlands	16	51.969443, 5.758940
		17	51.967693, 5.758896
		18	51.971671, 5.747826
Italy	Farms	19	42.914466, 13.854588
(San Benedetto del Tronto)		20	42.944809, 13.859857
		21	42.943098, 13.853856
	Peri-urban	22	42.883455, 13.879388
		23	42.951012, 13.850783
		24	42.934424, 13.891933
	Wetlands	25	42.896600, 13.911895
		26	42.899042, 13.909813
		27	42.903365, 13.908667

Mosquito identifications

All female mosquitoes were identified to species level, following the European identification key for female mosquitoes (Becker *et al.*, 2010). The number of *Cx. pipiens* mosquitoes captured each month was not sufficient to statistically test for temporal differences in biotype and hybrid ratios. Therefore, all 190 *Cx. pipiens* females available for analysis from Sweden, and a selection of 300 *Cx. pipiens* females from Italy and 299 *Cx. pipiens* females from The

Netherlands were used, resulting in a total of 789 mosquitoes analysed. Samples from The Netherlands and Italy were partially random selected with 100 samples per habitat, for both countries.

Selected mosquitoes were further identified to species (*Cx. pipiens* or *Cx. torrentium*) and biotype (*pipiens, molestus,* or hybrid) level. We followed the real-time PCR assay for differentiation between the *Cx. pipiens* biotypes as described in detail before (Vogels *et al.*, 2015). Briefly, for *Cx. pipiens* we used forward and reverse primers Cx_pip_F (5'-GCGGCCAAATATTGAGACTTTC-3') and Cx_pip_R (5'-ACTCGTCCTCAAACATCCAGACATA-3'). For identification of biotype *molestus* we used probe Cpp_mol_P (5'-FAM-TGAACCCTCCAGTAAGGTA-MGB-3'), and for biotype *pipiens* we used the two probes Cpp_pip_P1 (5'-VIC-CACACAAAYCTTCACCGAA-MGB-3') and Cpp_pip_P2 (5'-VIC-ACACAAACCTTCATCGAA-MGB-3'). Hybrids were identified when both probes for biotype *pipiens* and *molestus* were amplified by real-time PCR. For identifications of *Cx. torrentium* we used forward and reverse primers Cx_tor_F (5'-CTTATTAGTATGACACAGGACGACAGAAA-3') and Cx_tor_R (5'-GCATAAACGCCTACGCAACTACTAA-3'), and probe Cx_tor_P (5'-FAM-ATGATGCCTGTGCTACCA-MGB-3'). Thermocycler conditions were 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 62°C for 1 min. The PCR was run on the CFX96 Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA) and data were analysed in CFX manager 2.0 (Bio-Rad Laboratories, Hercules, CA).

Statistical analyses

Main effects (trap type, country, and habitat) and within-effects (habitats within each country, and country within each habitat) on the ratios of *Cx. pipiens* mosquitoes were tested with Pearson's Chi-square tests. Significant effects were further evaluated with pairwise comparisons and corrected with the Bonferroni correction. All data were analysed in the statistical software package R (R Development Core Team, 2016).

Results

In total 5,202 *Cx. pipiens* females were collected of which 3,878 females were collected with the BGS trap and 1,324 females with the MMLP trap (Table 2). Of the 789 mosquitoes selected for analysis, 663 mosquitoes were identified as *Cx. pipiens*, of which 463 (69.8%) were identified as biotype *pipiens*, 127 (19.2%) as biotype *molestus*, and 73 (11.0%) as hybrids (S1 Dataset). In addition, 14 mosquitoes were identified as *Cx. torrentium*, which all originated from Sweden. The number of *Cx. torrentium* mosquitoes was too low for reliable statistical tests, and these samples were therefore excluded from further analyses. The remaining 112 mosquitoes did not amplify a PCR product.

Table 2. Total number of collected *Cx. pipiens* females per trap type, habitat, and country. BGS = Biogents Sentinel trap, MMLP = Mosquito Magnet Liberty Plus trap, SW = Sweden, NL = The Netherlands, and IT = Italy.

	BGS			MMLP		
	SW	NL	IT	SW	NL	IT
Farms	19	252	128	29	64	21
Peri-Urban	56	1063	111	44	451	37
Wetlands	33	969	1247	24	101	553
Total	108	2284	1486	97	616	611

Both trap types, BGS and MMLP, trapped similar ratios of the *Cx. pipiens* biotypes and hybrids (χ^2=2.35, df=2, p=0.31; Fig. 1A). Thus, data from both trap types were pooled for further analyses.

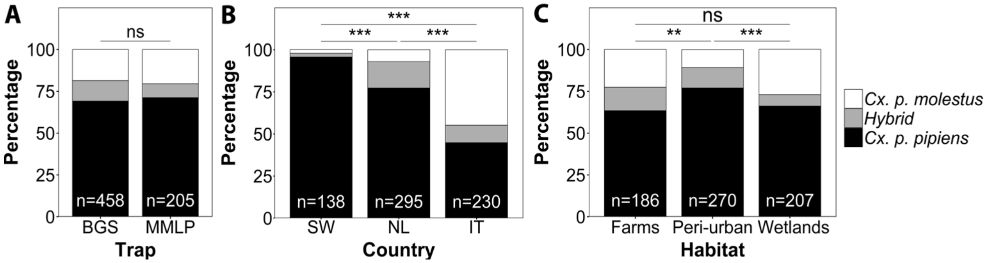

Figure 1. Main effects of (A) trap type, (B) country, and (C) habitat on the ratio of *Culex pipiens* biotypes and their hybrids. The total sample size (n) is indicated for each bar. Significance is displayed for each pairwise comparison, with ns = not significant, ** = *p*<0.01, *** = *p*<0.001. BGS = Biogents Sentinel trap, MMLP = Mosquito Magnet Liberty Plus, SW = Sweden, NL = The Netherlands, and IT = Italy.

The ratios of *Cx. pipiens* biotypes and hybrids were significantly different between the three countries in Europe (χ2=173.62, df=4, *p*<0.001; Fig. 1B). Pairwise comparisons between countries showed that *Cx. pipiens* ratios were different between each combination of Italy, The Netherlands, and Sweden (all pairwise comparisons: *p*<0.001). The proportion of biotype *pipiens* was highest in Sweden (90%) and gradually decreased towards more southern latitudes, with the lowest proportion of biotype pipiens in Italy (40%).

The ratios of *Cx. pipiens* biotypes and hybrids were also significantly different between habitat types (χ2=26.59, df=4, *p*<0.001; Fig. 1C). Peri-urban habitats had a relatively higher proportion of biotype pipiens compared to both farms (*p*<0.01), and wetlands (*p*<0.001). There was no difference in ratios between farms and wetlands (*p*=0.16).

In order to gain more insight in the interaction between country and habitat, pairwise comparisons were made between the habitats within each country, and the countries within each habitat type (Fig. 2). Ratios of *Cx. pipiens* biotypes and hybrids were significantly different between habitats in Italy (χ2=25.05, df=4, *p*<0.001) and The Netherlands (χ2=26.37, df=4, *p*<0.001), but were similar within Sweden ($\chi 2$=6.11, df=4, *p*=0.19; Fig. 2). In The Netherlands, farms were different due to the relatively high proportion of biotype *molestus* and hybrids (*p*<0.01), whereas in Italy wetlands were different due to the high proportion of biotype *molestus* (*p*<0.001).

Ratios of *Cx. pipiens* biotypes and hybrids were significantly different between countries within each of the habitat types (*p*<0.001; Fig. 2). Farms in Sweden had a relatively higher proportion of biotype *pipiens* compared to Italy (*p*<0.001) and The Netherlands (*p*<0.01), which both had relatively more biotype *molestus* and hybrids. For peri-urban habitats, ratios were significantly different among all countries (pairwise comparisons: p≤0.001), with a gradual increase of biotype *pipiens* towards northern latitudes. Wetlands in Italy had a relatively low proportion of biotype *pipiens* but high proportion of biotype *molestus,* compared to The Netherlands (*p*<0.001) and Sweden (*p*<0.001), which both had relatively high proportions of biotype *pipiens*.

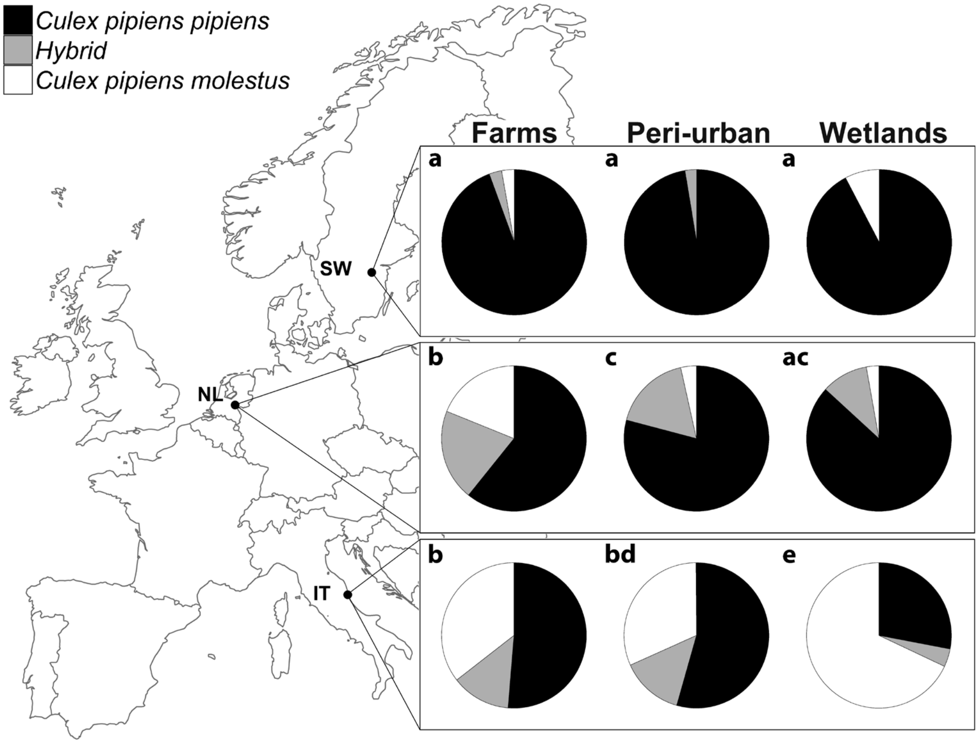

Figure 2. Within-effect of habitat within each of the three countries on the ratio of *Culex pipiens* biotypes and their hybrids (rows), and within-effect of country within each habitat type (columns). The sample size for each pie chart ranges between n=26-115. Letters display significant differences between ratios shown in rows and columns, at a significance level of *p*<0.05. SW = Sweden, NL = The Netherlands, and IT = Italy.

Discussion

The aim of this study was to assess the relative abundance of the *Cx. pipiens* biotypes and their hybrids in different habitats from northern to southern latitudes in Europe, using two trap types. We found a strong latitudinal effect on the ratios of the *Cx. pipiens* biotypes and hybrids, with a gradient of decreasing biotype *pipiens* from northern to southern latitudes. Habitat types also influenced the ratios of *Cx. pipiens* biotypes and hybrids, but effects were not consistent at the different latitudes.

Due to low numbers, *Cx. torrentium* was excluded from the analyses in this study. All *Cx. torrentium* females that we identified originated from Sweden, whereas no *Cx. torrentium* females were found in The Netherlands and Italy. These results are consistent with previous studies that showed a relatively high abundance of *Cx. torrentium* in northern European countries (Hesson *et al.*, 2011; Hesson *et al.*, 2013), and relatively low abundance or even absence in southern Europe (Di Luca *et al.*, 2016).

No difference was found between the ratio of *Cx. pipiens* biotypes and their hybrids collected with the BGS and MMLP traps. Despite the differences in trapping mechanism between the two traps, there was no apparent difference in the attraction of the biotypes and their hybrids towards both traps. For studies focusing on relative abundance both traps can thus be used equally well, but differences in the total numbers of collected *Cx. pipiens* mosquitoes between both traps do exist. Results of studies on mosquito abundance with different traps may not be directly comparable.

Our study shows the sympatric occurrence of both *Cx. pipiens* biotypes and hybrids in aboveground habitats throughout Europe. These results are in line with previous findings from The Netherlands (Vogels *et al.*, 2015), Germany (Rudolf *et al.*, 2013), Austria (Zittra *et al.*, 2016), Portugal (Gomes *et al.*, 2009; Osório *et al.*, 2014), Italy (Di Luca *et al.*, 2016), and Greece (Gomes, Kioulos, *et al.*, 2013). There was a clear gradient of decreasing biotype *pipiens* and increasing biotype *molestus* proportions from northern to southern latitudes. In Sweden the major part of the *Cx. pipiens* mosquitoes collected consisted of the *pipiens* biotype, whereas ratios in Italy were more equal between *pipiens* and *molestus* biotypes. This pattern was visible when ratios were determined per country without differentiating between the different habitat types, as well as when ratios for each country were split over the three habitat types. Previously, only a single record of the *molestus* biotype was known for Sweden (Schäfer & Lundström, 2001). In addition, the proportion of hybrids in Sweden was much lower compared to The Netherlands and Italy, which can be explained by the near absence of biotype *molestus* in all habitat types in Sweden. In this study we confirm that both biotypes and their hybrids occur aboveground at latitudes up to 58°24’36”N.

All peri-urban habitats combined had a relatively higher proportion of biotype *pipiens* and fewer biotype *molestus* compared to farms and wetlands. This pattern was, however, not consistent when comparing habitats within each of the three countries. In Sweden the ratios were similar for the three habitats, whereas in The Netherlands farms and in Italy wetlands ratios were different from the other two habitat types. This inconsistency could be explained by differences in, for instance, climate, microhabitat, availability of breeding sites, and hosts which all may influence the presence of the biotypes. These factors are likely to differ more between countries at different latitudes than between nearby habitats at one geographic location. Our findings also show variation at the local scale between habitats in The Netherlands and Italy. Especially the Italian wetlands stand out because of the high proportion of biotype *molestus*. Although a higher proportion of biotype *pipiens* was expected in such bird-rich habitats, a previous study showed high proportions of biotype *molestus* up to 82% in both urban and rural habitats with aboveground breeding sites in Italy (Di Luca *et al.*, 2016). The same study also showed high variation in *Cx. pipiens* biotype composition throughout Italy (Di Luca *et al.*, 2016). The ratios that we found for each country do, therefore, not represent an overall ratio of *Cx. pipiens* for the entire country, but rather for the specific sampling location. The sampling strategy used in our study is suitable for direct comparisons between locations at different latitudes, due to the consistent design over all countries. Studies that place traps at random locations throughout a country are more useful to get insight in local variation and dynamics of *Cx. pipiens* within a country (Rudolf *et al.*, 2013; Osório *et al.*, 2014; Vogels *et al.*, 2015; Di Luca *et al.*, 2016; Zittra *et al.*, 2016).

Cx. pipiens populations that are dominated by biotype *pipiens* play an important role in the natural transmission cycle of WNV in birds, whereas the risk of WNV outbreaks among humans is increased in populations with high levels of hybridization (Fonseca *et al.*, 2004). Up to now, outbreaks of WNV among humans have only occurred in southern and central Europe, including Italy (Rizzoli *et al.*, 2015). The overall proportion of hybrids is higher in The Netherlands than Italy, which is not consistent with the more equal proportion of biotypes in Italy than in The Netherlands. This indicates a more complex cause of hybridization than solely density dependence. If WNV would get established in The Netherlands, the higher degree of hybridization may result in a higher likelihood of bridging of WNV from birds to humans. However, other factors such as vector competence and climate determine whether transmission cycles can get established. Such factors are most likely limiting the transmission of WNV in northern Europe (Fros *et al.*, 2015; Vogels *et al.*, 2016). Future studies on vector competence of the *Cx. pipiens* biotypes and hybrids under different climatic scenarios are needed in order to gain more insight in the risk of the transmission of viruses by mosquitoes in Europe.

Conclusions

The BGS and MMLP traps collected equal ratios of the *Cx. pipiens* biotypes and their hybrids. A clear gradient of decreasing biotype *pipiens* and increasing biotype *molestus* proportions from northern to southern latitudes in Europe was found. Hybrids were found in all countries, but highest proportions were recorded in The Netherlands and Italy. Furthermore, *Cx. pipiens* ratios between habitat types were different. These differences were, however, not consistent when comparing habitat types within countries. Future research should focus on, (i) vector competence of *Cx. pipiens* biotypes and hybrids at different latitudes in Europe to assess the risks of WNV transmission in northern Europe, and (ii) the ecology of hybrids in order to estimate the risk of WNV being transmitted to humans**.**

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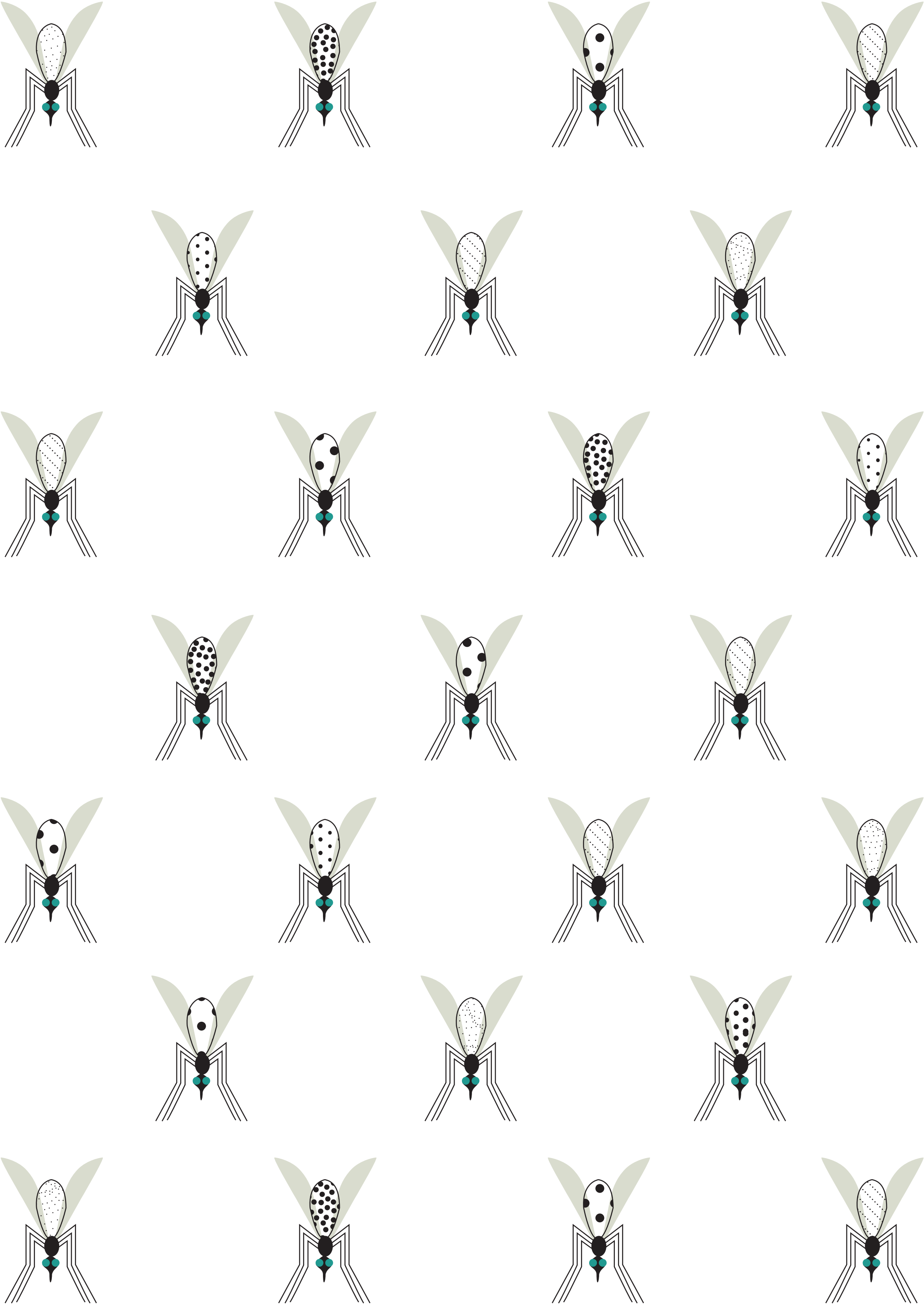
Supplementary

S1 Dataset. Results of *Cx. pipiens* biotype analyses.
doi: 10.1371/journal.pone.0166959.s001



Chapter 4

Community analysis of the abundance and diversity of biting midge species (Diptera: Ceratopogonidae) in three European countries at different latitudes

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Abstract

Background: The outbreaks of bluetongue and Schmallenberg disease in Europe have increased efforts to understand the ecology of *Culicoides* biting midges and their role in pathogen transmission. However, most studies have focused on a specific habitat, region, or country. To facilitate wider comparisons, and to obtain a better understanding of the spread of disease through Europe, the present study focused on monitoring biting midge species diversity in three different habitat types and three countries across Europe.

Methods: Biting midges were trapped using Onderstepoort Veterinary Institute light traps at a total of 27 locations in Sweden, The Netherlands and Italy, comprising farm, peri-urban and wetland habitats. From July 2014 to June 2015 all locations were sampled monthly, except for during the winter months. Trapped midges were counted and identified morphologically. Indices on species richness, evenness and diversity were calculated. Community compositions were analysed using non-metric multidimensional scaling (NMDS) techniques.

Results: A total of 50,085 female midges were trapped during 442 collection nights. More than 88% of these belonged to the Obsoletus group. The highest midge diversity was found in Sweden, while species richness was highest in The Netherlands, and most specimens were trapped in Italy. For habitats within countries, diversity of the trapped midges was lowest for farms in all countries. Differences in biting midge species communities were more distinct across the three countries than the three habitat types.

Conclusions: A core midge community could be identified, in which the Obsoletus group was the most abundant. Variations in vector communities across countries imply different patterns of disease spread throughout Europe. How specific species and their associated communities affect disease risk is still unclear. Our results emphasize the importance of midge diversity data at community level, how this differs across large geographic range within Europe, and its implications on assessing risks of midge-borne disease outbreaks.

Abbreviations

NMDS: Non-metric multidimensional scaling; AHSV: African horse sickness virus; BTV: bluetongue virus; SBV: Schmallenberg virus; OVI; Onderstepoort Veterinary Institute; CORINE: Coordination of information on the environment; IIKC: Interactive Identification Key for *Culicoides*

Keywords: *Culicoides*, midge sampling, species diversity, OVI trap, community ecology

Introduction

Worldwide around 1400 species of *Culicoides* (biting midges, Diptera: Ceratopogonidae) have been described (Mehlhorn *et al.*, 2007). At present, the European Interactive *Culicoides* Key (Mathieu *et al.*, 2010; Mathieu *et al.*, 2012) includes 110 species. A minority of these species has thus far been described as important vectors for arthropod-borne viruses (arboviruses) (Mellor *et al.*, 2000; Carpenter *et al.*, 2006). Biting midges from the *Culicoides* Obsoletus group (De Liberato *et al.*, 2005; Savini *et al.*, 2005; Carpenter *et al.*, 2008; Elbers *et al.*, 2013; Koenraadt *et al.*, 2014; Meiswinkel *et al.*, 2014), *C. imicola* Kieffer, 1913 (Goffredo & Meiswinkel, 2003), *C. pulicaris* (Linnaeus, 1758) (Purse *et al.*, 2004; Takken & Knols, 2007; Koenraadt *et al.*, 2014), and *C. punctatus* (Meigen, 1804) (Takken & Knols, 2007; Carpenter *et al.*, 2009; Hoffmann *et al.*, 2009; Wilson & Mellor, 2009; Ander *et al.*, 2012; Balenghien *et al.*, 2014; Meiswinkel *et al.*, 2014) are important vectors occurring in Europe. Of the over 50 viruses isolated from biting midges, several are of major international significance (Mellor *et al.*, 2000). Animal diseases caused by viruses such as Akabane virus, bovine ephemeral fever virus, African horse sickness virus (AHSV) and bluetongue virus (BTV) are all transmitted by biting midges. Both infection with AHSV and bluetongue are of such international significance that they are listed by the World Organisation for Animal Health (OIE) as focus diseases that can have serious socio-economic or public health consequences, and are of major importance for international trade (World Organisation for Animal Health, 2016).

Outbreaks of midge-borne viruses in Europe, e.g. BTV since 2006, and more recently Schmallenberg virus (SBV) during 2011–2013 (Wernike *et al.*, 2012; Gubbins *et al.*, 2014), have had a great impact on the European livestock sector (Van Schaik *et al.*, 2008; Saegerman *et al.*, 2014; Veldhuis *et al.*, 2014). Milk production by infected livestock is often reduced, and the virus affects unborn calves and lambs when the mother becomes infected during gestation, resulting in non-viable offspring. These impacts, in addition to livestock movement restrictions, result in considerable economic losses. Factors such as intensified transportation of livestock and the rise in global temperature may further increase the risk of arbovirus outbreaks in Europe. Rising temperatures create opportunities for vector populations to increase rapidly, and allow viruses to complete their extrinsic incubation period in vectors faster, which both imply an increased potential for pathogen transmission (Wilson & Mellor, 2008).

Although research following the outbreaks of BTV and SBV has improved our knowledge of the ecology of *Culicoides* biting midges, these studies often focus on a specific habitat, region, or country (Fassotte *et al.*, 2008; Kiel *et al.*, 2009; Kaufmann, Steinmann, *et al.*, 2012; Venail *et al.*, 2012; González *et al.*, 2013). Therefore, it is difficult to make direct comparisons among the results of these studies. Thus, to facilitate wider comparisons on biting midge communities at European level, we aimed to simultaneously sample midge species distribution, abundance, and diversity in three habitat types within three representative countries at different latitudes in Europe, where large differences in environmental characteristics could be expected.

Materials and Methods

Midge trapping

Adult midges were trapped using Onderstepoort Veterinary Institute (OVI) light traps. A 30 cm 8 W fluorescent black light tube was used to attract midges (Mehlhorn *et al.*, 2007). When in close proximity of the trap, midges were sucked in by the down-draught fan, which was powered by the main grid or a 12 V, 24–32 Ah battery (Venter & Meiswinkel, 1994; Takken & Knols, 2007; Ander *et al.*, 2012). The top of the trap was placed at a height of 1.5–2 m and traps were at least 100 m apart to prevent interference between them. The collection bucket had a capacity of 500 ml and larger insects were excluded by polyester netting (mesh size 2–4 mm) placed around the light source of the trap. The bucket was filled with 50 ml water-soap solution.

Sampling procedures

Traps were placed in three countries at different latitudes: Sweden (surroundings of Linköping 58°24’38.9”N, 15°37’17.5”E), The Netherlands (surroundings of Wageningen 51°57’53.3”N, 5°39’46.4”E), and Italy (surroundings of San Benedetto del Tronto 42°56’58.1”N, 13°52’42.6”E). Within each country, farm, peri-urban and wetland habitats were selected. Selection criteria for habitat type and trap location have been described in (Möhlmann *et al.*, 2017). In brief, each habitat type was represented by three unique sampling locations. Traps were positioned in these locations within 50 m of open stables of dairy cattle (‘farm’), a residential property (‘peri-urban’) or standing water (‘wetlands’). Habitat types mostly matched the classification of the CORINE European Land cover database (EEA, 2000).

From July 2014 to June 2015, except for the months December, January and February (and March for Sweden), monthly collections were performed for six consecutive days in each of the countries. Traps were active for 24 h and were emptied and rotated among the sampling locations between 08:00 h and 17:00 h the next day. Midges were sorted and stored at -20 °C in Eppendorf tubes containing 70% ethanol solution.

Sample identification

All female midges were identified to species level in collections that contained less than 100 individual midges. For collections that contained more than 100 midges (14% of the collections), a random sub-sample of at least 50 individuals was identified as an estimation of the species composition of the total collection. All identifications were performed using the Interactive Identification Key for *Culicoides* (IIKC) (Mathieu *et al.*, 2010; Mathieu *et al.*, 2012). Morphologically similar species were recorded as belonging to a group or complex (Nolan *et al.*, 2007; Harrup *et al.*, 2015).

Statistical analyses

Species diversity indices were calculated with the Simpson's Index of Diversity: $1 - D = 1 - \frac{\sum n_i(n_i-1)}{N(N-1)}$, Shannon-Wiener Diversity Index: $H' = -\sum_{i=1}^{R} p_i \ln(p_i)$, and the Shannon-Wiener evenness: $E = \frac{H'}{\ln(S)}$. Diversity indices were calculated for each of the three countries as well as for farm, peri-urban and wetland habitats. To better understand whether sufficient trapping efforts had been made for a reasonable estimate of species diversity, a rarefaction curve of the species and the number of trapped midges was created with the rarecurve function within the *vegan* version 2.9.2. (Oksanen *et al.*, 2009) package in R version 3.2.3. (R Development Core Team, 2015).

Non-metric multidimensional scaling (NMDS) analyses were used to evaluate the combined effects of country, habitat and diversity on the midge community composition (Young *et al.*, 1995). An NMDS analysis can deal with abundant null measures in a dataset and calculates a reliable best model fit for shortest distances between the elements. The degree of stress calculated within this analysis indicates the reliability of the plot that is generated with NMDS, whereby lower stress corresponds to a higher reliability of the plot. For values above 0.3 the NMDS ordination plot is considered arbitrary. For NMDS analyses, the metaMDS function with the Bray-Curtis dissimilarity metric was used. All data were analysed using the statistical software package R version 3.2.3. (R Development Core Team, 2015).

Results

A total of 442 trap collections were performed in Sweden, The Netherlands and Italy (Table 1). In 305 (69%) of these collections one or more biting midges were trapped, whereas in the remainder of the collections (31%), no biting midges were trapped. A total of 50,729 biting midges were trapped during this study. Of these specimens, 7818 (15.4%) female midges were identified to species level. After identification, a total of 50,085 female (98.7%) biting midges were estimated to be trapped. Other individuals either were males or damaged to the extent that they could not be identified morphologically. A total of 45 midge species were found for the three countries combined. Of all female biting midges trapped during the field study, the number of specimens trapped was highest for the Obsoletus group (88.6%), followed by *C. punctatus* (2.3%), *C. pulicaris* (2.2%) and *C. festivipennis* (Kieffer 1914) (1.6%).

The rarefaction curves for each of the three countries are beyond their exponential growth and start to level off (Fig. 1). Although more sampling efforts would increase the number of species expected to be found (mostly in Sweden and The Netherlands), we believe that our sampling effort was sufficient for obtaining a representative number of species for the three countries.

Table 1. Midge species diversity. Estimators of taxonomic diversity with values for the Simpson's Index of Diversity, Shannon-Wiener diversity and Shannon-Wiener evenness for three habitats (farms, peri-urban and wetlands) in three countries (Sweden, the Netherlands and Italy).

	Sweden				the Netherlands				Italy				Total
Taxonomic diversity	Farm	Peri-urban	Wetland	Total	Farm	Peri-urban	Wetland	Total	Farm	Peri-urban	Wetland	Total	
No. of specimens trapped	3267	46	761	4074	8270	185	3530	11985	33682	124	220	34026	50085
No. of samples	42	48	46	136	52	53	50	155	47	51	53	151	442
No. of species trapped	15	12	14	18	18	14	29	35	12	11	14	20	45
Simpson Index of Diversity	0.640	0.821	0.786	0.723	0.439	0.828	0.777	0.596	0.036	0.715	0.748	0.051	0.285
Shannon-Wiener diversity	1.439	2.000	1.801	1.714	0.807	2.051	1.863	1.386	0.127	1.589	1.750	0.178	0.813
Shannon-Wiener evenness	0.531	0.805	0.682	0.593	0.279	0.777	0.553	0.390	0.051	0.663	0.663	0.060	0.214

Although the lowest numbers of samples and specimens were trapped in Sweden, the highest species diversity was found there. The lowest values of species diversity were found in Italy, but most specimens were trapped in this country (Table 1). The lowest midge species diversity was found at farms in all countries. The highest midge species diversity was found for midges trapped in peri-urban habitats in Sweden and The Netherlands, while the midge diversity was almost similar for peri-urban and wetlands in Italy (Table 1). Catches from peri-urban habitats had the lowest number of specimens, while the highest number of specimens were trapped at farms in all countries.

From the 4074 female midges trapped in Sweden, 18 species were identified. The most common species were from the Obsoletus group (47%), *C. pulicaris* (22%) and *C. achrayi* (Kettle & Lawson, 1955) (10%) (Table 2). The dominating species among the 3,267 females trapped on farms were from the Obsoletus group (54%) and *C. pulicaris* (27%). From the 46 female midges trapped in peri-urban habitats, *C. kibunensis* (Tokunaga, 1937) (28%) and *C. vexans* (Staeger, 1839) (22%) were the most common, whereas the 761 specimens from wetlands in Sweden were dominated by *C. achrayi* (31%) and *C. festivipennis* (27%).

The Netherlands had the highest species richness with 35 species identified among 11,985 female midges trapped during the study period. The most common species were from the Obsoletus group (78%), *C. punctatus* (8%) and *C. festivipennis* (5%). For wetland and farm habitats the most abundant species were comprised of those species. The 185 midges trapped from peri-urban habitats were dominated by *C. kibunensis* (31%) and *C. brunnicans* (Edwards, 1939) (16%).

Of the 20 species trapped in Italy, the Obsoletus group was by far the most dominant with 97% of all 34,026 trapped female midges. The trapped midges from farm (33,682) and peri-urban (124) habitats were dominated by the Obsoletus group, although *C. pulicaris* (1%) and *C. duddingstoni* Kettle & Lawson, 1955 (27%) were also trapped more than other species in the two habitats, respectively. From the 220 midges trapped in Italian wetland habitats, *C. circumscriptus* Kieffer, 1918 (44%) and *C. submaritimus* Dzhafarov, 1962 (18%) were most abundant.

Table 2. Midge species abundance. List of midge species with number of specimens for each country (Sweden, the Netherlands and Italy) and habitat type (farms, peri-urban and wetlands).

	Sweden				the Netherlands				Italy				Total
Species list	Farm	Peri-urban	Wetland	Total	Farm	Peri-urban	Wetland	Total	Farm	Peri-urban	Wetland	Total	
C. achrayi	173	1	235	409	7	20	23	50	41	2	1	44	503
C. alazanicus							283	283		2		2	285
C. albihalteratus		1		1									1
C. brunnicans					4	30		34					34
C. cameroni	4	1	8	13	1		6	7			5	5	25
C. caucoliberensis							1	1		1		1	2
C. chiopterus	34	1		35	2001	15	119	2135					2170
C. circumscriptus			1	1	5		3	8	45	7	96	148	157
C. derisor							7	7					7
C. dewulfi					4			4					4
C. duddingstoni									116	34	18	168	168
C. fagineus	40		30	70									70
C. fasciipennis					3		81	84					84
C. festivipennis	42	1	207	250	10	10	524	544	2	4	1	7	801
C. flavipulicaris									54			54	54
C. griseidorsum									6	15	5	26	26
C. grisescens					163		8	171					171
C. heliophilus							1	1					1
C. heteroclitus									85			85	85
C. impunctatus	14		1	15		1		1					16
C. indistinctus							8	8					8
C. jurensis							1	1					1
C. kibunensis	64	13	67	144	2	58	207	267					411
C. longipennis							2	2			1	1	3
C. lupicaris	86	3	8	97	1			1	37			37	135
C. manchuriensis						2	7	9					9
C. maritimus										2	21	23	23
C. newsteadi					1	1	4	6					6
C. nubeculosus							6	6					6
Obsoletus group	1726	9	136	1871	5860	28	1329	7217	33,069	54	25	33,148	42,236
C. pictipennis		2	20	22		3	78	81					103
C. picturatus							5	5					5
C. poperinghensis	21		4	25	4		4	8					33
C. pseudopallidus											1	1	1
C. pulicaris	892	1	6	899	11		18	29	184	1		185	1113
C. punctatus	120	3	36	159	191	7	776	974	38	2	1	41	1174
C. reconditus						1	1	2					2
C. remmi											1	1	1
C. riethi	1			1	2		3	5					6
C. salinarius	16			16			5	5					21
C. simulator							18	18					18
C. subfasciipennis						5		5					5
C. submaritimus											39	39	39
C. tauricus							1	1					1
C. vexans	34	10	2	46		4	1	5	5		5	10	61

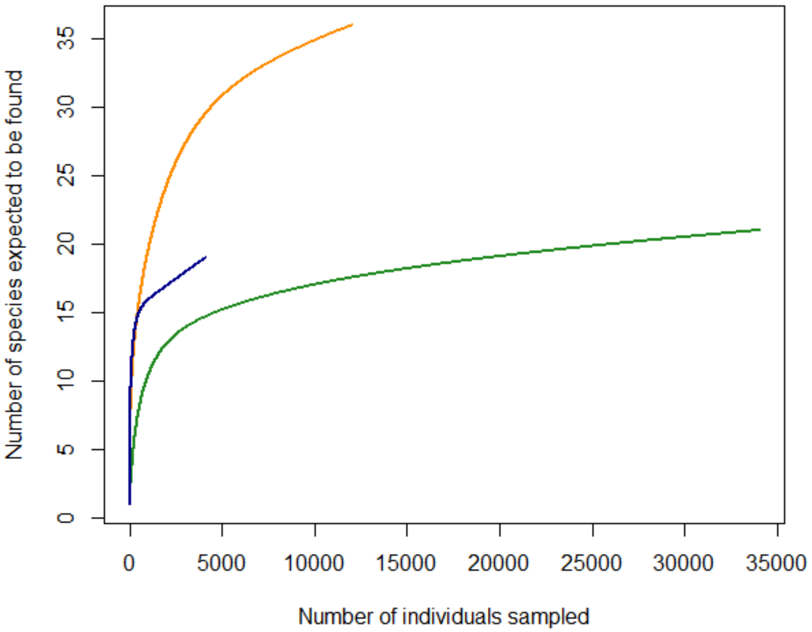


Figure 1. Rarefaction plot of sampling effort. The plot shows the number of species expected to be found for the number of individuals sampled for Sweden (blue), Italy (green) and The Netherlands (orange).

Of all midge species trapped, 38% (17/45) were unique to one of the three habitat types. Three species occurred exclusively at farms, two species in peri-urban habitats and 12 species only in wetland habitats (Fig. 2). Figure 3 shows that more than half of the 45 species identified were trapped in only one of the countries (26/45, 58%), while 20% (9/45) of the species were trapped in all three countries. This core community included the most abundant species from the three countries: the Obsoletus group, *C. punctatus*, *C. pulicaris*, *C. festivipennis* and *C. achrayi.*

Combining the presence and abundance of different midge species trapped for the different countries and habitat types into one statistical analysis provides additional information about vector communities associated with specific regions. Dissimilarity matrices resulting from NMDS analyses show clear differences in biting midge community composition among the areas investigated in countries at different latitudes (p=0.002, stress-value=0.084) (Fig. 4a). Differences in communities were not found among habitats (p=0.976, stress-value=0.084) (Fig. 4b). However, looking at habitats within each country, midge communities were found to be different for some of the habitats (Fig. 4c). Midge communities among Dutch (p=0.048, stress-value=0.081) and Italian (p=0.040, stress-value=0.0594) habitats were significantly different from each other. For habitats in Italy this difference was mainly driven by the wetland habitat (Fig. 4c). Midge communities among habitats in Sweden were comparable (p=0.577, stress-value=0.099).

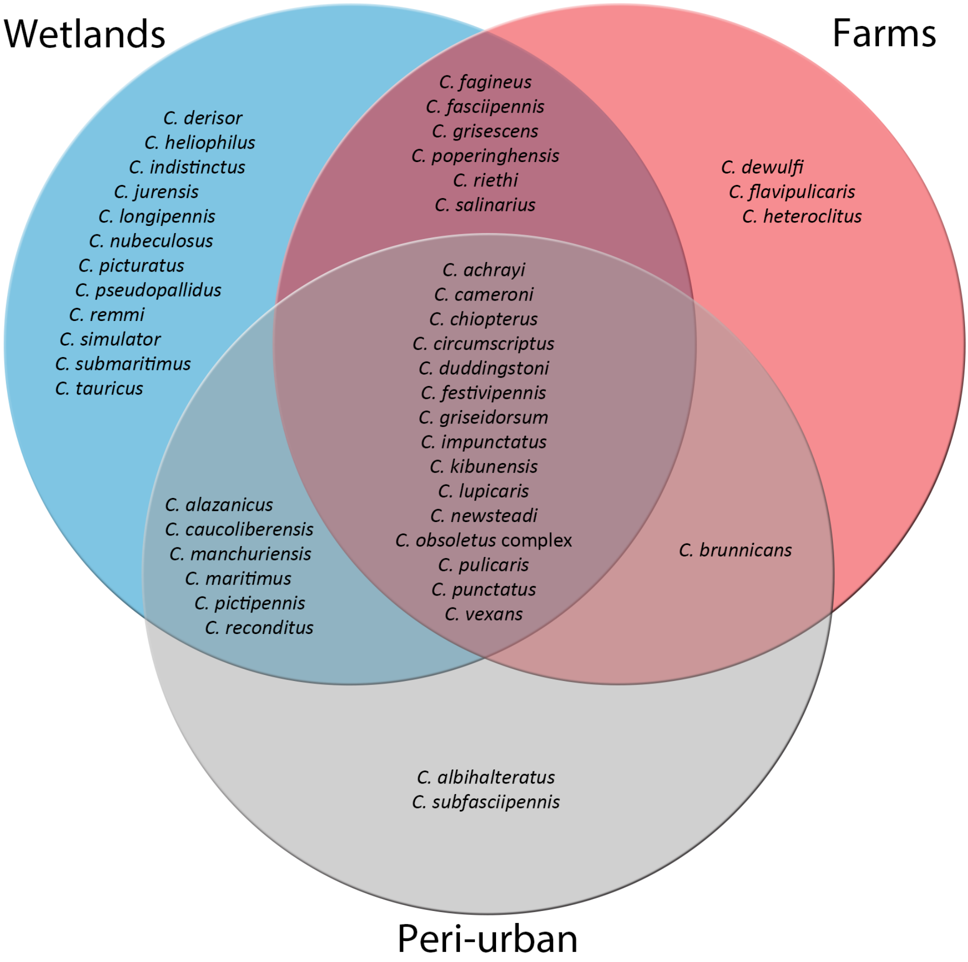

Wetlands
Farms
Peri-urban
C. derisor
C. heliophilus
C. indistinctus
C. jurensis
C. longipennis
C. nubeculosus
C. picturatus
C. pseudopallidus
C. remmi
C. simulator
C. submaritimus
C. tauricus
C. fagineus
C. fasciipennis
C. grisescens
C. poperinghensis
C. riethi
C. salinarius
C. dewulfi
C. flavipulicaris
C. heteroclitus
C. achrayi
C. cameroni
C. chiopterus
C. circumscriptus
C. duddingstoni
C. festivipennis
C. griseidorsum
C. impunctatus
C. kibunensis
C. lupicaris
C. newsteadi
C. obsoletus complex
C. pulicaris
C. punctatus
C. vexans
C. alazanicus
C. caucoliberensis
C. manchuriensis
C. maritimus
C. pictipennis
C. reconditus
C. brunnicans
C. albihalteratus
C. subfasciipennis

Figure 2. Venn diagram of habitats. Diagram shows the absolute presence of midge species found in farm (red), peri-urban (grey) and wetland (blue) habitats.

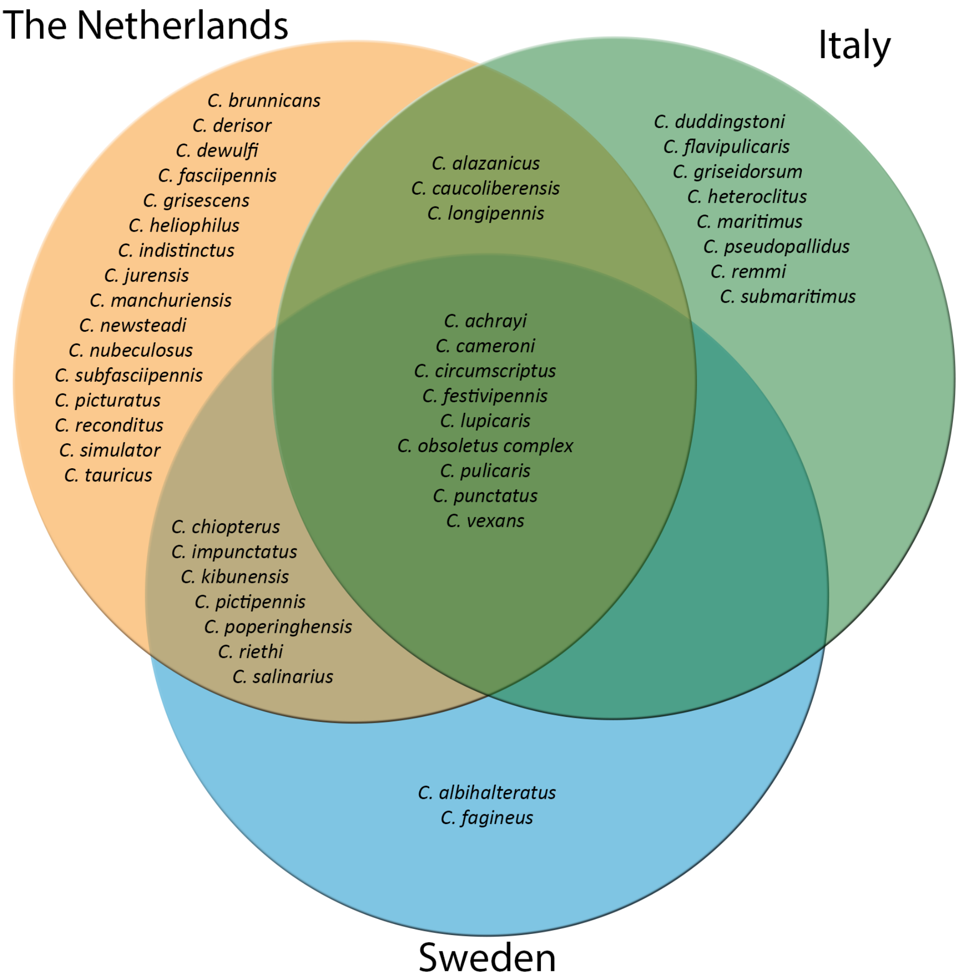

The Netherlands
Italy
Sweden
C. brunnicans
C. derisor
C. dewulfi
C. fasciipennis
C. grisescens
C. heliophilus
C. indistinctus
C. jurensis
C. manchuriensis
C. newsteadi
C. nubeculosus
C. subfasciipennis
C. picturatus
C. reconditus
C. simulator
C. tauricus
C. alazanicus
C. caucoliberensis
C. longipennis
C. duddingstoni
C. flavipulicaris
C. griseidorsum
C. heteroclitus
C. maritimus
C. pseudopallidus
C. remmi
C. submaritimus
C. achrayi
C. cameroni
C. circumscriptus
C. festivipennis
C. lupicaris
C. obsoletus complex
C. pulicaris
C. punctatus
C. vexans
C. chiopterus
C. impunctatus
C. kibunensis
C. pictipennis
C. poperinghensis
C. riethi
C. salinarius
C. albihalteratus
C. fagineus

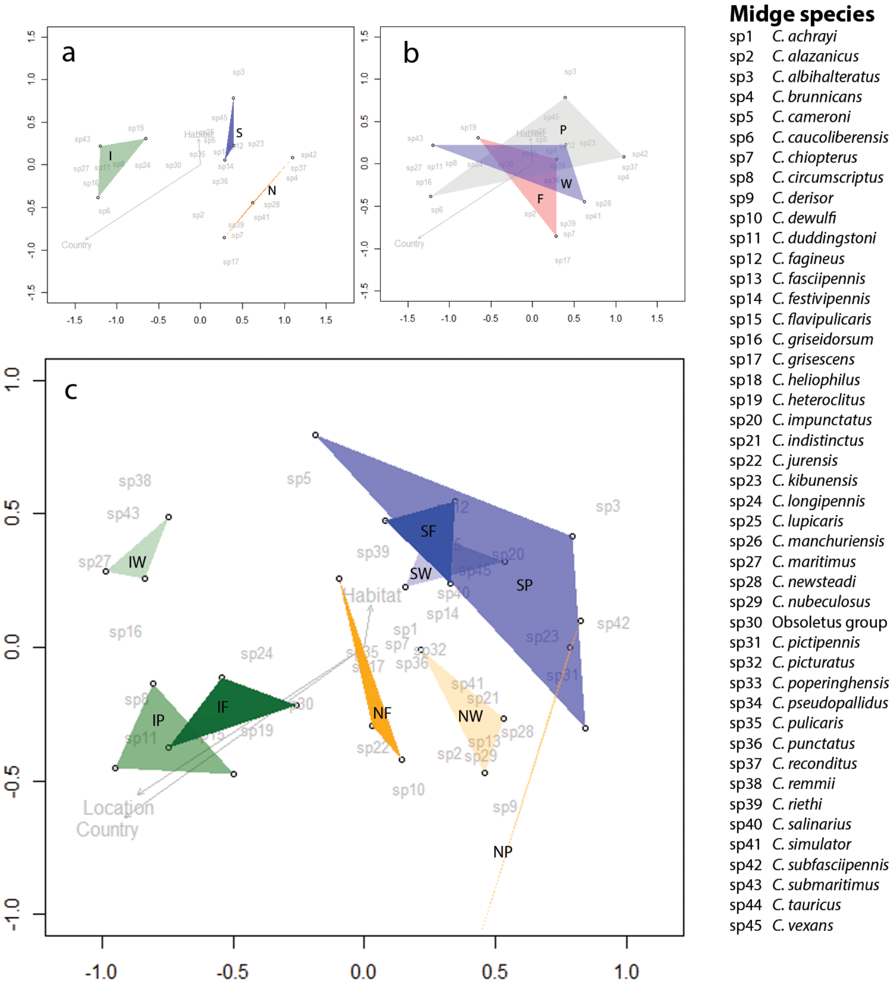
Figure 3. Venn diagram of countries. Diagram shows the absolute presence of midge species found in Sweden (blue), The Netherlands (orange) and Italy (green).

Discussion

Biting midge community composition clearly differed among the areas we investigated at different latitudes. This is illustrated by the diversity indices (Table 1), which was highest in Sweden, followed by The Netherlands, and then Italy. In addition, the Venn-diagram (Fig. 3) shows that 57% of the trapped midge species were found in only one of the countries. Finally, the dissimilarity matrix (Fig. 4a) distinguishes distinct midge communities among countries. Although communities varied among the areas investigated for the countries, a core community of midges seems to be present nevertheless (Fig. 3). This core community includes the five most abundant species from the three countries: the Obsoletus group, *C. punctatus*, *C. pulicaris*, *C. festivipennis* and *C. achrayi.* While this core community occurs throughout Europe and across different habitats, it cannot be assumed that their contribution to disease spread is similar in all countries as temperature, interaction with other (host) species, and genetic variation within midge species (Jones & Foster, 1974; Mellor *et al.*, 2000) also vary throughout Europe. However, these known midge vector species are present in a core community throughout Europe, and with rising temperature as a consequence of climate change, and continued increase in animal transport, it is expected that disease outbreaks will increase throughout Europe. Increasing temperature will simultaneously affect the rapid increase of midge vector populations, and at the same time allow viruses to complete their extrinsic incubation period in vectors faster, which both imply an increased potential for pathogen transmission (Wilson & Mellor, 2008).

While community composition clearly differed among the sampled areas at different latitudes, communities were similar among habitat types (Fig. 4a, b). However, when differentiating habitats within countries, there were marked habitat effects on community composition (Fig. 4c). Habitat communities from farm, wetland, and peri-urban sites differed within The Netherlands and Italy, while communities in Sweden were more similar to each other. These results are comparable to diversity and community composition found for mosquitoes in Europe (Möhlmann *et al.*, 2017). Both mosquito and biting midge communities show clear differences among areas in the three representative countries, while these communities are different for habitats only within the countries studied. This suggests that local habitat factors can be important for vector community composition, but that ecological factors at large geographical distances between sites have a more significant impact.

Some of the biting midge species that were morphologically identified, were thus far not known to be present in the studied countries. Of 18 midge species identified for Sweden, two species (*C. cameroni* and *C. fagineus*) could not be confirmed by the IIKC (Mathieu *et al.*, 2010) or literature (Nielsen & Kristensen, 2011; Ander *et al.*, 2012) (see Additional file 1: Table S1 for an overview). For The Netherlands, 12 species (*C. brunnicans*, *C. cameroni*, *C. caucoliberensis*, *C. derisor*, *C. indistinctus*, *C. jurensis*, *C. longipennis*, *C. manchuriensis*, *C. picturatus*, *C. reconditus*, *C. simulator* and *C. tauricus*) were not earlier described (Elbers & Meiswinkel, 2014; Meiswinkel *et al.*, 2014; Nederlandsesoorten.nl, 2018), and for Italy three species (*C. achrayi*, *C. cameroni* and *C. vexans*) were not found in literature (Gomulski *et al.*, 2006; Foxi *et al.*, 2011) or distribution maps of the IIKC. Because most of these species are known to be present in countries surrounding the countries studied here, we expect that the distribution is correct but was simply not confirmed before. We will continue to work with these samples and confirm the findings with barcoding techniques before adding them to current distribution lists.



Midge species

sp1	*C. achrayi*
sp2	*C. alazanicus*
sp3	*C. albihalteratus*
sp4	*C. brunnicans*
sp5	*C. cameroni*
sp6	*C. caucoliberensis*
sp7	*C. chiopterus*
sp8	*C. circumscriptus*
sp9	*C. derisor*
sp10	*C. dewulfi*
sp11	*C. duddingstoni*
sp12	*C. fagineus*
sp13	*C. fasciipennis*
sp14	*C. festivipennis*
sp15	*C. flavipulicaris*
sp16	*C. griseidorsum*
sp17	*C. grisescens*
sp18	*C. heliophilus*
sp19	*C. heteroclitus*
sp20	*C. impunctatus*
sp21	*C. indistinctus*
sp22	*C. jurensis*
sp23	*C. kibunensis*
sp24	*C. longipennis*
sp25	*C. lupicaris*
sp26	*C. manchuriensis*
sp27	*C. maritimus*
sp28	*C. newsteadi*
sp29	*C. nubeculosus*
sp30	Obsoletus group
sp31	*C. pictipennis*
sp32	*C. picturatus*
sp33	*C. poperinghensis*
sp34	*C. pseudopallidus*
sp35	*C. pulicaris*
sp36	*C. punctatus*
sp37	*C. reconditus*
sp38	*C. remmii*
sp39	*C. riethi*
sp40	*C. salinarius*
sp41	*C. simulator*
sp42	*C. subfasciipennis*
sp43	*C. submaritimus*
sp44	*C. tauricus*
sp45	*C. vexans*

Figure 4. Results of NMDS analyses. Panel a: Figure shows midge community compositions for Sweden (S), The Netherlands (N) and Italy (I). Panel b: NMDS analysis for farms (F), peri-urban (P) and, wetland (W) habitats based on number of midges trapped per species in each habitat and country. **c** NMDS analysis based on number of midges trapped per species at each location in each country and habitat (Sweden in blue: SF, SP and SW, The Netherlands in orange: NF, NP and NW, Italy in green: IF, IP and IW). The Bray-Curtis dissimilarity index was used to determine dissimilarities among midge community compositions. Stress-value=0.084 for panels a and b, which indicates a very good fit of the model. Stress-value=0.216 for panel c, which indicates a suspect fit of the model.

From the European core midge community identified in this study, at least three species are (potential) vectors of pathogens. The Obsoletus group was the most abundant trapped in all countries, especially in farm habitats. Species in this group are known to transmit both

BTV and SBV (De Liberato *et al.*, 2005; Savini *et al.*, 2005; Carpenter *et al.*, 2008; Elbers *et al.*, 2013; Koenraadt *et al.*, 2014; Meiswinkel *et al.*, 2014), and considered the most important midge vector species in Europe. However, the Obsoletus group consists of several species [*C. chiopterus* (Meigen, 1830; *C. dewulfi* Goetghebuer, 1936; *C. obsoletus* (Meigen, 1818) (*s.s.*); *C. scoticus* Downes & Kettle, 1952; and *C. montanus* Shakirzjanova, 1962] for which morphological identification is difficult and very laborious (Nielsen & Kristensen, 2011). *Culicoides* identification remains a challenge, especially for specific groups or complexes of species (Mathieu *et al.*, 2012; Harrup *et al.*, 2015). As new techniques such as DNA (barcode) sequencing and MALDI TOF (matrix assisted laser desorption/ionisation time of flight) mass spectrometry (Kaufmann *et al.*, 2011; Kaufmann, Schaffner*, et al.*, 2012; Kaufmann, Steinmann*, et al.*, 2012; Harrup *et al.*, 2015) become available for identification, it will be easier to process large numbers of specimens. However, correct reference databases, morphological identifications, and the link with ecology remain essential components in *Culicoides* research. Therefore, the European Interactive Identification Key for *Culicoides* (IKCC) developed by Mathieu *et al.* (Mathieu *et al.*, 2010) is a useful tool to obtain accurate morphological identifications. Those species that are difficult or impossible to separate by morphological identification, such as the Obsoletus group, can be further identified with molecular tools. Although species in the Obsoletus group are recognized as important potential vectors (Gomulski *et al.*, 2005)2005, it remains unknown what the species-specific (within the Obsoletus group) contribution to pathogen transmission is. Priority should therefore be given to investigate the Obsoletus group composition in more detail, to better understand disease dynamics.

The other two species found in this study that are (potential) vectors of pathogens were *C. punctatus* and *C. pulicaris.* These species were trapped in similar numbers and are known to transmit BTV and SBV (Purse *et al.*, 2004; Takken & Knols, 2007; Carpenter *et al.*, 2009; Hoffmann *et al.*, 2009; Wilson & Mellor, 2009; Ander *et al.*, 2012; Balenghien *et al.*, 2014; Koenraadt *et al.*, 2014; Meiswinkel *et al.*, 2014). Both species were found in all habitats and countries, although *C. punctatus* was mainly found in Dutch wetlands, while *C. pulicaris* was mostly present at farms in Sweden and Italy. One of the most important European BTV vectors for southern Europe, *C. imicola*, was not trapped during this study. Our trapping sites in Italy were further north compared to the known distribution of *C. imicola* (Calistri *et al.*, 2003), and results can therefore not be extrapolated for the most southern parts of Europe where *C. imicola* is present.

Although our sampling effort was comprehensive, as can be deduced from the rarefaction plot (Fig. 1), the study was carried out in a relatively limited area. *Culicoides* diversity found in our trappings is, therefore, not representative for the countries as a whole. In addition, trapping with a single trap type may not accurately represent midge fauna diversity (Elbers & Meiswinkel, 2014; Elbers *et al.*, 2016; Meiswinkel & Elbers, 2016). Nevertheless, because of the consistent study design and use of the same trap type, results can be compared among the three areas in each country and habitats in this study. Midge species and their abundance can be under- or over-estimated compared to the biting rate on livestock animals (Viennet *et al.*, 2011). Although mostly female biting midges were trapped, these were most likely not host-seeking midges, as they were attracted by a UV-light source (Elbers & Meiswinkel, 2015). The exact attraction mechanism for female midges towards the trap is thus far unknown. Nonetheless, the OVI trap seems to be the only effective midge trap currently available (Probst *et al.*, 2015), and this stresses the need for appropriate monitoring methods against biting midges.

A study in Germany showed that adult midges can be trapped during winter months (Hoffmann *et al.*, 2009). Although midge numbers captured in our traps were reduced in the months just before and after the winter period, it is not clear whether midges ceased their activity in our study. In addition to their activity during winter, the larval habitats of known midge vectors have not been extensively investigated (Foxi & Delrio, 2010; Harrup *et al.*, 2013; Zimmer *et al.*, 2013; Zimmer *et al.*, 2014). With more knowledge on the habitats for both *Culicoides* larvae and adults, it will be possible to better understand the factors influencing differences in communities among countries and habitats.

Although the current study revealed differences in biting midge communities among habitats within countries, the underlying factors for this were not identified. Biting midge species diversity was surprisingly high in peri-urban habitats (Table 1), possibly as only few specimens of different species were captured in comparable numbers in this habitat type. In contrast, diversity was lowest at farms in all three countries, which could be explained by the high abundance of the Obsoletus group. Several of the farm collections had high abundance of midges and were therefore sub-sampled for identification. A few individuals of new species may be found if all individuals of farm samples would be identified. However, testing this scenario by simulating additional species in our dataset did not change our conclusions on diversity indices and the dominance of the Obsoletus group in farm habitats. The overwhelming abundance of this species group in farm habitats suppresses the influence on diversity measures of other species occurring on farms. Possibly, specific larval or adult habitats are present on farms that cause species from the Obsoletus group to flourish, while other species may not take advantage of these habitats.

Chaves *et al.* (2011) suggested that lower diversity of vector communities is expected to increase the risk of amplification and spread of a vector-borne disease, because lower vector species diversity is thought to be correlated with higher relative abundance of some species within the community. This is in line with our findings, since the country (Italy) and habitat (farms) with the lowest diversity indices had the highest abundance of midges from the Obsoletus group. On the other hand, a theoretical study by Roche *et al.* (2013) suggested that greater vector species richness leads to higher abundance, and can therefore amplify pathogen transmission. These authors showed that specific vector species in these complex community dynamics could be essential in epidemic take-off, even if these vectors are weakly susceptible to pathogen infection. Given that vector-borne diseases would require a set of multiple species that together influence the rate of transmission, understanding the species composition of vector communities and their interactions with pathogens is becoming increasingly important. Ultimately, entomological field data should be used for the development of mathematical and statistical models, to more accurately assess the effect of environmental factors on midge population dynamics and how this influences disease risks.

Conclusions

A core European midge community could be identified, with important disease vector species from the Obsoletus group, *C. punctatus* and *C. pulicaris*, as the most abundant in this core community. The presence of a core community throughout Europe suggests that disease dynamics can be homogenous, since the core community is present in all countries and habitats. However, in-depth analysis of the complete midge community composition showed that differences were more distinct among countries than habitat types. In other words, although some midge species are found throughout Europe, distinctive communities can be found within each country. This suggests that specific species within countries may impose a more heterogeneous disease dynamics than expected when only looking at the core community. Although we have elucidated how species communities differ among countries and habitats, it is still unclear how these specific species and their associated communities affect disease risk.

Acknowledgements

We would like to thank the students that helped collecting the field data; Rickard Pettersson from Sweden and Mahmod Hamdan from Italy. Farmers, nature area managers and home-owners in Sweden, The Netherlands and Italy that provided us with the essential locations needed for this research are acknowledged for their support. We also thank Marcel Dicke for providing comments on a previous version of this manuscript.

Supplementary

Additional file 1: Table S1. A list of the species per country found in this study. References to previous research of *Culicoides* fauna in the same countries are made, as well as remarks on the distribution of the species as described in the IIKC.

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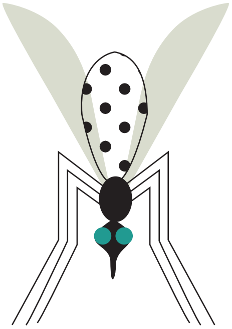

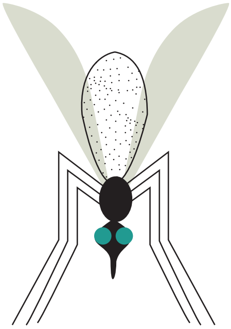

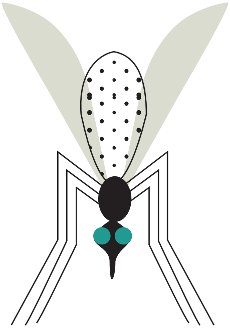

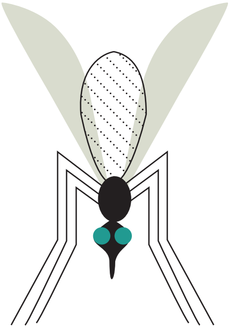

Chapter 5

Latitudinal diversity of biting midge species within the Obsoletus group across three habitats in Europe

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Abstract

Culicoides species from the Obsoletus group are important vectors of bluetongue and Schmallenberg virus. This group consists of several species that cannot easily be identified using morphological characteristics. Therefore, limited information is available about their distribution and habitat preferences. In this study, we aimed to elucidate the species composition of the Obsoletus group in three habitat types at climatically different latitudes across Europe. Traps were placed in three habitat types in three countries at different latitudes. After DNA extraction, biting midges were identified using PCR and gel electrophoresis. Extraction of DNA using Chelex proved to be a cost and time efficient method for species identification. A latitudinal effect on the relative abundance of species from the Obsoletus group was found. Species composition was unique for most country-habitat combinations. The majority of biting midges were either *C. obsoletus* s.s. or *C. scoticus*, and both species were found at all latitudes and habitats. Their wide distribution and their high abundance at livestock farms make these species likely candidates for rapid farm-to-farm transmission of pathogens throughout Europe. Our results emphasize the need to differentiate Obsoletus group species, to better understand their ecology and contribution to pathogen transmission.

Keywords: *Culicoides*, bluetongue, Obsoletus complex, Onderstepoort light trap, livestock disease, Schmallenberg, species composition, vectors

Introduction

When bluetongue and Schmallenberg viruses rapidly spread throughout central, western, and even northern European countries, it became clear that not only *Culicoides imicola* (Kieffer, 1913) was responsible for virus transmission. While *C. imicola* was known to transmit bluetongue virus in African and south-European countries (Venter *et al.*, 1998; De Liberato *et al.*, 2003), this species was not found in central or northern parts of Europe (Mellor & Wittmann, 2002). Subsequently, several species of *Culicoides* were identified as potential vectors in areas outside the distribution range of *C. imicola*. These species included *C. newsteadi* Austen, 1921 (Foxi *et al.*, 2016), *C. punctatus* Meigen 1804 (Hoffmann *et al.*, 2009), species of the Pulicaris complex (Caracappa *et al.*, 2003), and species of the Obsoletus group (De Liberato *et al.*, 2005; Elbers *et al.*, 2013). Although species in the Obsoletus group are considered the most important vectors of the aforementioned viruses in northern and central Europe, it remains unclear which species within this group is responsible for most of the transmission.

The term “Obsoletus group” is cladistically artificial because not all species are part of a monophyletic group. However, in *Culicoides* literature it is commonly used to indicate a collection of related and morphologically similar species. In Europe, the Obsoletus group consists of at least five related species, namely *C. chiopterus* (Meigen, 1830), *C. dewulfi* (Goetghebuer, 1936), *C. montanus* (Shakirzjanova, 1962), *C. obsoletus* sensu stricto (s.s.) (Meigen, 1818), and *C. scoticus* (Downes & Kettle, 1952) (Harrup *et al.*, 2015; Goffredo *et al.*, 2016). Although identification of these species is possible by morphological characteristics (Nielsen & Kristensen, 2011; Goffredo *et al.*, 2016), it remains a challenge even for biting midge taxonomists. This is especially the case for three species that together form the Obsoletus complex; *C. obsoletus* s.s., *C. scoticus* and *C. montanus* (Harrup *et al.*, 2015). Because *C. montanus* has only been found in the most southern parts of Europe and not in the area under study in this paper (Goffredo *et al.*, 2016), we refer to the Obsoletus complex as *C. obsoletus* s.s. and *C. scoticus* throughout this paper. These two species are nearly impossible to separate based on morphological characteristics. Identification of the species within the Obsoletus group and complex is therefore more reliable with molecular tools (Lehmann *et al.*, 2012). Unfortunately, identification of species in the Obsoletus group is not consistently performed when studying *Culicoides* vectors in the field (De Liberato *et al.*, 2005; Foxi *et al.*, 2016). This makes it difficult to compare outcomes of different studies and, at times of disease outbreaks, to understand which species within the group are most responsible for pathogen transmission.

Species of the Obsoletus group are frequently associated with livestock, and therefore often found in large numbers at livestock farms (Elbers & Meiswinkel, 2015; Steinke *et al.*, 2016; Möhlmann *et al.*, 2018). However, each species of the Obsoletus group seems to have its own breeding habitat and/or host preference. While *C. chiopterus* and *C. dewulfi* prefer cow dung as larval habitat, species of the Obsoletus complex (*C. obsoletus* s.s. and *C. scoticus*) have been found breeding in a much wider range of substrates (Steinke *et al.*, 2016). Adult females of all species in the Obsoletus group seem to prefer larger livestock animals as host, although *Obsoletus* s.s. was found to have a broader host range, and to readily bite humans and birds (Viennet *et al.*, 2013). When female biting midges take a blood meal from either cattle, sheep or horses, females of *C. chiopterus* favour biting on the legs, whereas members of the Obsoletus complex (*C. obsoletus* s.s., *C. scoticus*) and *C. dewulfi* prefer the head, back, and flanks (Elbers & Meiswinkel, 2015). These ecological and behavioural differences suggest that we cannot consider these biting midge species as a homogenous group.

Differences between these species become even more apparent when comparing their vector competence. During outbreaks of bluetongue disease and Schmallenberg disease, several studies collected *Culicoides* from the field and tested them for virus infection. Different studies identified bluetongue virus in pools of the Obsoletus group, *C. dewulfi*, *C. chiopterus*, and *C. scoticus* (De Liberato *et al.*, 2005; Foxi *et al.*, 2016). Schmallenberg virus was also detected in the four most common species of the Obsoletus group (*C. chiopterus, C. dewulfi, C. obsoletus* s.s., and *C. scoticus*). During the 2011 outbreak of Schmallenberg in The Netherlands, Elbers *et al.* (2013) found infection rates of 0.14% for *C. chiopterus*, 0.05% for *C. obsoletus* s.s., and 0.47% for *C. scoticus*. In the same year infection rates of 0.44% for *C. chiopterus*, 1.06% for *C. obsoletus* s.s., and 1.10% for *C. dewulfi* were found in Belgium (Regge *et al.*, 2012). Other studies showed that infection rates of Schmallenberg virus can reach up to 2.20% for biting midges from the Obsoletus group (Rasmussen *et al.*, 2012), although no distinction was made between the species in these two studies.

Differences in larval habitat and host preference, in combination with an almost ten-fold variation in infection rates among species of the Obsoletus group, emphasize the importance of knowing how these species are distributed in habitats throughout Europe. We therefore investigated their distribution and relative abundance in three habitat types (farms, peri-urban, wetlands) for areas in three countries (Sweden, The Netherlands, and Italy) representing three different latitudes in Europe, using a standardized collection protocol for all locations. We expected species from the Obsoletus group to have specific ecological preferences for larval habitats, hosts, as well as climate. Therefore, we hypothesized that each habitat and country would represent a unique composition of Obsoletus group species.

Materials and Methods

Collection of *Culicoides*

Collection and identification of biting midges was earlier described in (Möhlmann *et al.*, 2018). In short, adult biting midges were collected using Onderstepoort Veterinary Institute (OVI) light traps with black light as attractant. Biting midges were sucked in by the downdraught fan, and collected in a small 500 ml bucket filled with 50 ml water-soap solution. Traps were rotated among 27 locations spread over Sweden (surroundings of Linköping N58.410808, E15.621532), The Netherlands (surroundings of Wageningen N51.964795, E5.662898), and Italy (surroundings of San Benedetto del Tronto N42.949483, E13.878503), i.e. nine locations per country, and three habitat types (farms, peri-urban, and wetlands) within each country. Three trap locations were selected for each habitat within a country. Trapping locations and their selection criteria were previously described (Vogels *et al.*, 2016; Möhlmann *et al.*, 2018). The three countries were selected for their different climate, each representing different latitudes across Europe. Farm locations were within 50 m of open dairy cattle stables, peri-urban locations close to residential property, and wetlands had standing water in the proximity. Traps were placed in the period from July 2014 to June 2015 except for the winter months December, January and February (and March for Sweden). Monthly collections were performed for six consecutive days in each of the countries. Traps were active for 24 hours and were emptied the next day. Biting midges were sorted and stored at -20 °C in Eppendorf tubes containing 70% ethanol solution.

Selection of samples

Female *Culicoides* biting midges were identified to species level with the use of the Interactive Identification Key for Culicoides (IIKC) (Mathieu *et al.*, 2010). In total, 50,085 female *Culicoides* biting midges were collected, of which an estimated 44,406 (89%) belonged to the Obsoletus group (Möhlmann *et al.*, 2018). From all available females of the Obsoletus group, a total of 628 was selected for molecular analysis (Table 1).

From the total dataset of Obsoletus group female biting midges, 100 individuals per habitat for each country were randomly selected. If less than 100 Obsoletus biting midges were available, all individuals were used. For Sweden, 208 biting midges of the Obsoletus group were analysed, for The Netherlands 243 biting midges, and for Italy a total of 177 biting midges (Table 1). Samples that did not show a result after PCR were excluded from the dataset (Table 1).

Table 1. Genetically identified females from the Obsoletus group.

	Farm	Peri-urban	Wetland	Total
Sweden	98/99	6/9	88/100	192/208
The Netherlands	98/100	41/43	99/100	238/243
Italy	99/100	49/53	23/24	171/177
Total	295/299	96/105	210/224	601/628

Total number genetically identified females from the Obsoletus group per habitat (farm, peri-urban, wetland) and country (Sweden, The Netherlands, Italy). Numbers on the right side of the backslash indicate the total number of individuals tested, whereas the numbers on the left side indicate those that could be positively identified after performance of the PCR.

Extraction methods

DNA was extracted from individual biting midges with two extraction methods. First, a standard extraction method with the DNeasy® Blood & Tissue Kit (Qiagen, Germany) was used based on an earlier identification protocol of Obsoletus group species (Lehmann *et al.*, 2012). The Animal Tissue Spin-Column protocol of the DNeasy® Blood & Tissue Kit was followed according to the manufacturer's instructions. In short, individual biting midges were dried on filter paper, placed in a 1.5 mL tube, quickly frozen in liquid nitrogen and subsequently crushed with a pestle. The sample was lysed for one hour, and purified DNA was eluted in 50 µL low-salt buffer. This extraction method was relatively costly and time consuming for the processing of many samples. We therefore decided to use a second extraction method based on Chelex (Miura *et al.*, 2017), that was more cost efficient and faster than the extraction with the commercial kit. For this protocol, 30 µL of 5% Chelex® 100 resin (143-2832 BioRad) in ultrapure water was added to each sample in a 96 well plate. After adding 2 µL 0.5 mg/mL Proteinase K (Ambion, The Netherlands), the samples were incubated at 56 °C for 24 hours, followed by 3 min at 99.9 °C in a PCR machine. Samples were subsequently centrifuged for 30 seconds at 4,700 rpm. Extracts from both methods were stored in the freezer at -20°C before further use.

Culicoides identification

For differentiation among species within the Obsoletus group the protocol as described by Lehmann *et al.* (2012) was used. Ingredients for the mastermix were adjusted for materials generally used for PCR in our laboratory. For amplification of the cytochrome c oxidase subunit I (COI) region, reverse primer PanCuli-COX1-727R (5'-TATAAACTTCDGGRTGNCCAAARAATC-3') and species specific forward primers: *C. dewulfi* dew-COI-fwd (5'-CGCCCGACATAGCATTCCCT-3'), *C. obsoletus* s.s. obs-COI-fwd (5'-CAGGAGCTTCTGTAGATTTGGCT-3'), *C. scoticus* sco-COI-fwd (5'-CCACAATTATTAATATGCGATCTACC-3'), and *C. chiopterus* chio-COI-fwd (5'-CCTTTATTTGTTTGGTCTGTTCTTC-3') were used. The mastermix for one sample consisted of 5 µL of 5X colourless reaction buffer, 6 µL of $MgCl_2$ (3mM), 5 µL dNTPs (1mM), 2 µL of the forward primer (10µM), 0.5 µL of every reverse primer (10µM), 0.125 µL GoTaq polymerase (5U/µL) (Promega, United States), 3.375 µL MilliQ, and 3 µL target DNA obtained from DNA extraction. The total volume of 25 µL was used for amplification with PCR settings on 15 min at 94°C, followed by 42 cycles of 30 s at 94°C, 45 s at 63°C, 45 s at 72°C, and a final step of 5 min at 72°C. Final temperature was kept at 4°C until samples were stored in the freezer at -20°C before further use.

PCR products (10 µL) were mixed with Orange G loading dye (5 µL) and loaded on a 1.5% agarose gel for electrophoresis for 45 minutes at 80 V. A 100 bp ladder was used as reference, as well as a negative control and positive controls for each of the four species. After electrophoresis, the gel was exposed to UV light in a Bio-Rad Gel Doc and imported into computer program Quintify One to visualise the bands. Species were identified according to differences in PCR product length whereby *C. dewulfi* was 468 bp, *C. obsoletus* s.s. 318 bp, *C. scoticus* 237 bp, and *C. chiopterus* 190 bp (Lehmann *et al.*, 2012).

The first PCR products from the expected four species of the Obsoletus group were excised from the agarose gel. They were subsequently recovered by the QIAquick Gel Extraction Kit (Qiagen), and sent to Eurofins Genomics (Ebersberg, Germany) for sequencing. The received COI sequences were assembled with Geneious and tested with the use of Nucleotide BLAST against the NCBI GenBank database. All COI samples were confirmed with at least 99% identity. The DNA extracts of these samples were diluted 1:1 and used as a positive control for the rest of the PCR identifications.

Statistical analyses

Main effects of country and habitat, and their within-effects (habitats within each country, and country within each habitat) on the ratios of *Culicoides* species within the Obsoletus group were tested with Fisher-Freeman-Halton test using the Chisq_test function with 9999 permutations in the COIN package version 1.1-3 (Hothorn *et al.*, 2008). Significant effects ($\alpha < 0.05$) were further evaluated with pairwise comparisons and corrected with the Bonferroni correction. All data were analysed in the statistical software program R version 3.2.3. (R Development Core Team, 2015).

Results

Of the 628 selected biting midges from the Obsoletus group, 42 (6.7%) were identified as *C. chiopterus*, 43 (6.8%) as *C. dewulfi*, 327 (52.1%) as *C. obsoletus* s.s., and 189 (30.1%) as *C. scoticus*. The remaining 27 (4.3%) biting midges did not yield a PCR product.

The ratios of species were significantly different among the three countries ($\chi^2 = 122.69$, df = 6, $p<0.001$; Fig. 1A), and the three habitat types ($\chi^2 = 36.03$, df = 6, $p<0.001$; Fig. 1B). Pairwise comparisons between countries showed that ratios of species were different for each comparison of the areas in Italy, The Netherlands, and Sweden (all pairwise comparisons: $p<0.001$). In Sweden, we found three species of the Obsoletus group (*C. dewulfi*, *C. obsoletus* s.s., and *C. scoticus*), compared to four in The Netherlands, and only the two species of the Obsoletus complex (C. *obsoletus* s.s. and *C. scoticus*) in Italy. Wetlands had relatively more Obsoletus complex biting midges, and less *C. chiopterus* and *C. dewulfi*, when compared to both farms ($p<0.01$) and peri-urban habitats ($p<0.001$). There was no significant difference in ratios between farms and peri-urban habitats ($p = 0.18$).

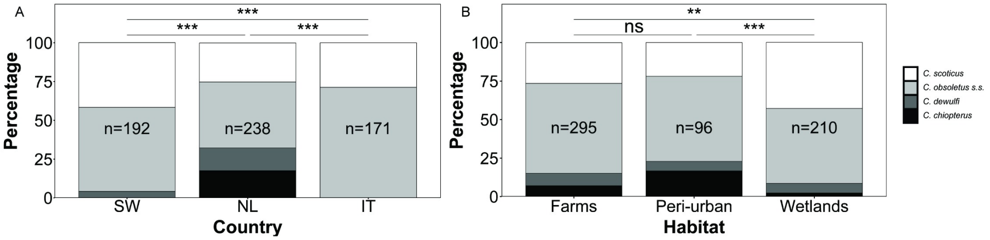

Figure 1. Main effects of (A) country and (B) habitat on the ratio of species in the Obsoletus group. The total sample size (n) is indicated for each bar. Significance is displayed for each pairwise comparison, with ns = not significant, ** = *p*<0.01, *** = *p*<0.001, SW = Sweden, NL = The Netherlands, and IT = Italy.

To get more insight into the interaction between country and habitat and its effects on species composition, pairwise comparisons were made between the habitats within each country, and between countries within each habitat type (Fig. 2). Pairwise comparisons between habitat types within each country showed that farms in Sweden were significantly different from peri-urban and wetland habitats (*p*<0.05; Fig. 2), due to the relatively high proportion of *C. obsoletus* s.s. and lower proportion of *C. scoticus*. In The Netherlands, wetland habitats were different from the other two habitat types. Dutch wetlands had relatively low proportions of *C. chiopterus* and *C. dewulfi* and were therefore different from farm and peri-urban habitats (*p*<0.001). In Italy, only farms were different from the wetland habitat (*p*<0.05), due to relatively high proportions of *C. scoticus* at farms and high proportions of *C. obsoletus* s.s. in wetlands (Fig. 2).

Ratios of the species were significantly different among countries within each of the three habitats (*p*<0.001; Fig. 2). In Sweden, the proportions of *C. scoticus* in peri-urban and wetland habitats were higher compared to The Netherlands, (*p*<0.01) and Italy (*p*<0.05). In The Netherlands the four Obsoletus group species had a more even distribution, differentiating this country from the other countries in all habitats (*p*<0.01). In Italy, relatively high proportions of *C. obsoletus* s.s. were recorded in all habitats, except when compared with Swedish farms which had relatively more *C. obsoletus* s.s. biting midges than Italian farms (*p*<0.05).

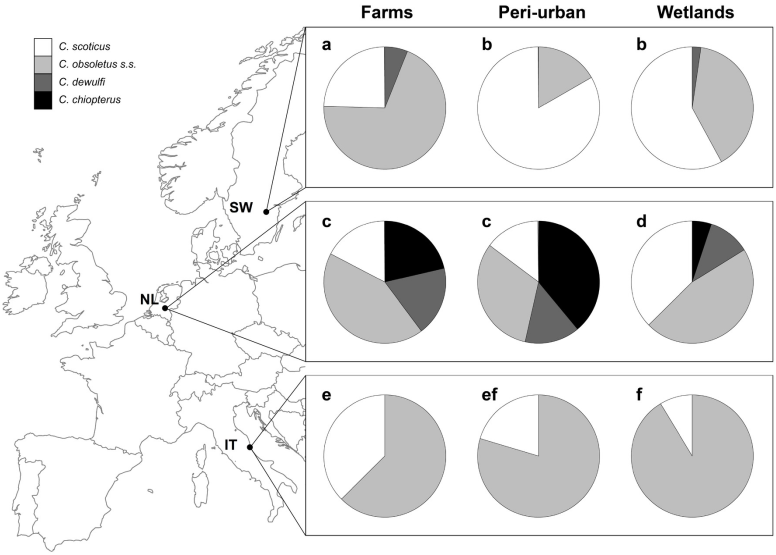

Figure 2. Within-effect of habitat in each of the three countries (rows) on the ratio of the four species in the Obsoletus group, and within-effect of country in each habitat type (columns). The sample size for each pie chart ranges from 6 to 99 (see also table 1). Letters display significant differences among ratios shown in rows and columns at a significance level of *p*<0.05. SW = Sweden, NL = The Netherlands, and IT = Italy.

Discussion

The aim of this study was to assess the distribution and relative abundance of biting midge species within the Obsoletus group in different habitats from northern to southern latitudes in Europe. We found a strong latitudinal effect on the relative abundance, as well as on the presence or absence of specific species of the Obsoletus group. Habitat types also influenced the ratios of species, but differences in relative abundance among habitat types were not consistent in areas at different latitudes.

Our study shows that the two species of the Obsoletus complex (*C. obsoletus* s.s. and *C. scoticus*) occur in at least three European countries in relatively high proportions. The other two species from the Obsoletus group were not found in each of the studied countries. *Culicoides dewulfi* was found in collections from both Sweden and The Netherlands, while *C. chiopterus* was only found in collections from The Netherlands. Neither of these two species were recorded in the collections from Italy. These results are in line with previous findings in Sweden, The Netherlands, and Italy that demonstrated that the two species of the Obsoletus complex are the dominant species in the Obsoletus group (Meiswinkel *et al.*, 2008; Nielsen *et al.*, 2010; Goffredo *et al.*, 2016; Magliano *et al.*, 2018). However, earlier research described the presence of the four species in each of the countries. In Sweden some individuals of *C. chiopterus* and *C. dewulfi* were found, but they only made up around 1% of the collections, and numbers were found to be lower at more northern latitudes (Nielsen *et al.*, 2010). Also in Italy, studies showed that *C. chiopterus*, *C. montanus*, and *C. dewulfi* can be occasionally caught, and these species made up a maximum of 3% of the total catches (Gomulski *et al.*, 2005; Goffredo *et al.*, 2016). The generally rare findings of *C. chiopterus* could be the result of biased sampling of this species in light traps. Yet our results do not support this, because at peri-urban habitats in The Netherlands, with the use of blacklight traps, we identified *C. chiopterus* as 39% of the collected biting midges.

Similar to our findings, *C. obsoletus* s.s. was more abundant than *C. scoticus* throughout Italy (Magliano *et al.*, 2018). Only in the most southern parts of Italy *C. scoticus* was relatively more abundant (Goffredo *et al.*, 2016). For The Netherlands, our finding on the relative abundance of the four species is comparable to earlier work (Meiswinkel *et al.*, 2008).

Nation-wide studies for each of the countries show that species of the Obsoletus group occur throughout Europe. This emphasizes that our collections were local, and cannot easily be extrapolated to country level. However, our study is the first to compare relative abundances of these species for three habitats at different latitudes with a controlled study design and with the same sampling effort for each location. A clear trend in all studies is that the Obsoletus complex is abundant in all three countries, but that countries in western Europe have relatively more individuals of *C. chiopterus* and *C. dewulfi* (Regge *et al.*, 2012). Both the Obsoletus complex, as well as *C. chiopterus* and *C. dewulfi* have been implicated as vectors for Schmallenberg virus (Regge *et al.*, 2012), although their contribution to pathogen spread remains unclear.

The problem of identification becomes even more relevant now that new (cryptic) species are being identified within the Obsoletus group (Gomulski *et al.*, 2005; Ander *et al.*, 2013; Meiswinkel *et al.*, 2015). Apparently, the Obsoletus group consists of even more genetically different species than just five. As long as we do not make a distinction between the species when research is conducted, either in the field or in laboratory vector competence studies, we will not fully understand how and by which species, biting midge-borne viruses could transmitted.

Molecular identification of many samples can be labour intensive and relatively expensive. In this study we show that alternative methods can be used. We first used the DNeasy Tissue Kit for DNA extraction, as described by Lehmann *et al.* (2012). We then tested a more cost efficient and high throughput method. DNA extraction with Chelex (see materials and methods) can be performed on whole biting midges or different body parts. In addition, it can be processed in 96-well plates and with less handling steps than earlier protocols. The results were consistent, and many more samples can be processed with the same time investment.

From the 628 biting midges used for molecular identification, 27 (4.3%) did not yield a PCR product. These could represent failed DNA extractions, or they could include uncharacterized new species (Ander *et al.*, 2013; Meiswinkel *et al.*, 2015). These samples could also be *C.*

montanus, as specific primers for this species were not included in the PCR. However, thus far *C. montanus* has only been identified in relatively low numbers in southern Italy (Goffredo *et al.*, 2016), and we therefore did not expect to find *C. montanus* in this study.

The majority of the identified biting midges was part of the Obsoletus complex (82.2%). In addition, the two species in the Obsoletus complex were found to be present in all three countries and in all habitats, which was also found in a study about spatial and temporal variation in biting midge species abundance across Europe (Cuéllar *et al.*, 2018). It has been suggested that *C. obsoletus* s.s. and *C. scoticus* are adapted to a wider range of habitats and are more resistant to extreme temperatures (Nielsen *et al.*, 2010). Furthermore, the availability of suitable hosts (horses, cattle, sheep, goats) for female biting midges, or that of larval breeding sites (i.e. dung, edges of ponds, marshes, tree holes) could influence the relative abundances of species found. Our study shows that the relative abundance of species was significantly different between farms and wetlands in all three areas studied, despite the relative close proximity of the habitats within each country. Apparently, species within the Obsoletus group differ in how well they can take advantage of these local habitats. However, species composition of farm and wetland habitats was not consistent among countries. Wetlands in Sweden and The Netherlands had relatively fewer *C. obsoletus* s.s., and more *C. scoticus* than farms, while this was the opposite for wetlands and farms in Italy. Although *C. scoticus* was found in relatively high abundance in wetlands in Sweden and The Netherlands, it should be noticed that absolute numbers of collected biting midges were 5 to 1300 times higher at farms when compared to wetlands (Möhlmann *et al.*, 2018). These high abundances of biting midges and their proximity to livestock animals make farms relatively favourable habitats for arbovirus transmission. Even though other biting midge species could play a role in maintaining a pathogen transmission cycle, *C. scoticus* and especially *C. obsoletus* s.s. are the most likely candidates for spread of a arbovirus at farms throughout Europe. It remains an open question whether their presence in relatively high abundance across all habitats and countries, is sufficient for virus spread from farm to farm. Other factors such as movement of livestock are likely to strongly influence large distance spread of a virus as well. Although the path of virus spread is not yet elucidated, our results show that at least two known biting midge vectors are present from northern to southern Europe in peri-urban areas, wetlands and livestock farms.

Conclusions

A strong country effect, indicative of latitudinal effects, on the relative abundance of species from the Obsoletus group was found. While the Obsoletus complex (*C. obsoletus* s.s., *C. scoticus*) was found at all latitudes, *C. chiopterus* was only identified in samples collected in The Netherlands, whereas *C. dewulfi* was not found among samples originating from Italy. Habitat types also influenced the ratios of species within the Obsoletus group, but effects were not consistent at different latitudes. Our suggestion to use a more efficient method for identification of Obsoletus group species may encourage others to also perform species identification, so that this becomes routine practice for *Culicoides* studies. The majority of the biting midges identified was part of the Obsoletus complex (82.2%), and both species were found at all latitudes and in all habitats. Their known susceptibility to viruses in combination with their wide distribution and high densities at livestock farms make *C. scoticus* and especially *C. obsoletus* s.s. likely candidates for rapid spread of midge-borne viruses throughout Europe.

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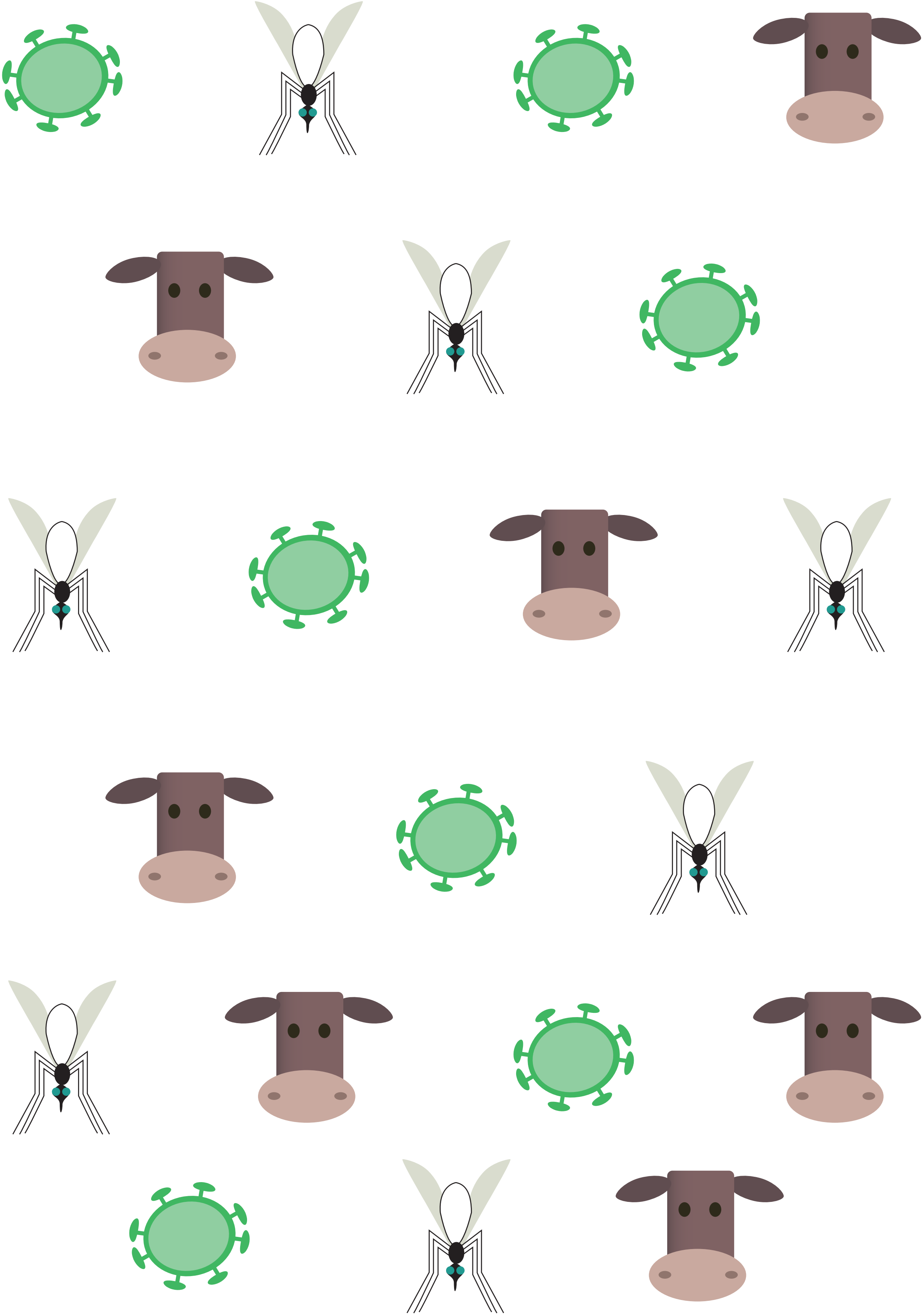

Chapter 6

Biting midge dynamics and bluetongue transmission: A multiscale model linking catch data with climate and disease outbreaks

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Submitted

Abstract

Bluetongue virus (BTV) serotype 8 has been circulating in Europe since a major outbreak occurred in 2006, causing economic losses to livestock farms. The unpredictability of the biting activity of midges that transmit BTV implies difficulty in computing accurate transmission models. This study uniquely integrates field collections of midges at a range of European latitudes (in Sweden, The Netherlands, and Italy), with a multi-scale modelling approach. We inferred the environmental factors that influence the dynamics of midge catching, and then directly linked predicted midge catches to BTV transmission dynamics. Catch predictions were linked to the observed prevalence amongst sentinel cattle during the 2007 BTV outbreak in The Netherlands using a dynamic transmission model. We were able to directly infer the bias between daily midge catch predictions and the true biting rate per cow per day. Compared to biting rate per cow per day the bias was around 50% of 24 hours midge catches with traps. Extending the estimated biting rate across Europe, for different seasons and years, indicated that whilst intensity of transmission is expected to vary widely from herd to herd, around 95% of naïve herds in western Europe have been at risk of sustained transmission over the last 15 years.

Introduction

Culicoides (Diptera: Ceratopogonidae) biting midges transmit a wide range of pathogens of veterinary importance worldwide including Akabane virus, bovine ephemeral fever virus, Schmallenberg virus, African horse sickness virus, epizootic haemorrhagic disease virus, and bluetongue virus (BTV) (Carpenter *et al.,* 2013; Carpenter *et al.,* 2015, *Purse et al.,* 2015). Historically, BTV was not endemic in Europe but there were sporadic incursions into the continent. However, in the past two decades various serotypes of BTV invaded southern Europe (Purse *et al.,* 2005). The outbreak of BTV serotype 8 near Maastricht, The Netherlands, in 2006 was the first BTV outbreak ever observed above latitude 50°N anywhere in the world (Toussaint *et al.,* 2006; Wilson & Mellor, 2009). The virus subsequently demonstrated the capacity to subsist throughout the winter period in north-western Europe, re-appearing amongst commercial livestock in 2007 (Saegerman *et al.,* 2008). Despite millions of vaccinations (Zientara & Sánchez-Vizcaíno, 2013), BTV serotype 8 re-emerged in France in 2015 (Sailleau & Viarouge, 2015), whilst other BTV serotypes remained circulating in southern Europe and caused periodic epizootic outbreaks in eastern European countries (Kyriakis *et al.,* 2015).

BTV causes economic losses in terms of livestock morbidity and mortality, the cost of surveillance and vaccination, as well as indirect costs caused by livestock movement restrictions that lead to agricultural business interruption (Pinior *et al.,* 2015). The persistence of BTV in Europe over the last two decades demonstrates the need to gain insight into the ecology of potential midge vector species across Europe as an aid to predict BTV transmission. Multiple biting midge species in Europe are known, or suspected to be, competent BTV vectors: species in the Obsoletus group (De Liberato *et al.,* 2005; Savini *et al.,* 2005; Carpenter *et al.,* 2008), *C. imicola* Kieffer, 1913 (Mellor, 1990), *C. pulicaris* (Linnaeus 1758) (Caracappa *et al.,* 2003), and *C. punctatus* (Meigen, 1804) (Hoffmann *et al.,* 2009).

Understanding the environmental factors that influence midge biting rates could result in more accurate predictions of BTV transmission, both within and between herds. Uncertainties about the biting behaviour of these midge species can be addressed by extensive field trapping and statistical analysis of trap catches. However, there is no consensus in the scientific literature on the most informative statistical method for analysing midge catch data. Published analyses of midge catch data include: discriminant analysis of seasonal midge abundance (Cuéllar *et al.,* 2018; Versteirt *et al.,* 2017; Baylis *et al.,* 2002), logistic regression of midge occurrence/absence (Calvete *et al.,* 2009), linear regression on log-maximum midge catches (Kluiters *et al.,* 2013), mixed effect Poisson regression on daily midge catches (Sanders *et al.,* 2011; Diarra *et al.,* 2015), as well as analyses combining such statistical methods (Searle *et al.,* 2013; Ducheyne *et al.,* 2013). Moreover, linking the outcomes of midge catch studies to a meaningful estimate of actual biting behaviour on commercial livestock has proven problematic. Midge biting rates for use in epidemiological modelling of BTV are typically estimated either by assuming some relationship to the true midge population size (Gubbins *et* *al.,* 2008; Hartemink *et al.,* 2009), or by assuming a relationship directly to the vector to host ratio (Guis *et al.,* 2012). However, recent comparative studies of different methods of assessing midges have found significant variation in catch quantities depending on the method used, e.g. between collection by aspiration directly from a host compared to using black light traps (Elbers & Meiswinkel, 2014), or sweep netting compared to black light attraction (Elbers & Meiswinkel, 2015). Therefore, estimates of midge biting intensity will rely heavily on the catch method and the location of the catching, e.g. animal or a trap near the barn. Understanding the link between midge surveillance and biting intensity has been identified as a critical knowledge gap in understanding midge-borne disease transmission (Koenraadt *et al.,* 2014; Mullens *et al.,* 2015).

The goal of this study is twofold: first, to establish a link between the expected number of midges trapped in 24h, to the expected number of bites received by a single cow over the same period. Second, to incorporate this link between catch number and biting rates into predictions of BTV risk across Europe in the recent past, focusing on within herd transmission. To achieve these goals we proceeded in four phases (see Fig. 1 for the workflow of this study), each of which depended on the outcome of the previous phases: (i) we made a series of field collections of midges using 24 hours of trapping at a range of latitudes, habitats, and days of the year, (ii) we performed a regression analysis for the climatic, seasonal, and environmental factors that influence the daily catching of known European BTV vectors, (iii) we inferred a bias of scale parameter between the midge catch regression model and the true midge biting rate using a comparison between BTV surveillance of sentinel cattle in The Netherlands in 2007 and the corresponding daily midge catch number predictions, (iv) we used the estimated true biting rate to map BTV risk across Europe over the last 15 years.

Materials and Methods

We have used a multi-scale modelling approach combining multiple modelling and inference types and techniques: generalised linear mixed-effect models (GLMMs), a mechanistic BTV transmission simulation model, marginal maximum likelihood inference, and direct calculation of resultant BTV risk predictions. We have parameterised our models with data from multiple existing sources (spatio-temporal climate time series, livestock density estimates, midge life process rates, BTV incidence time series), as well as data from our recent field study on the activity of midges at different latitudes in Europe (Möhlmann *et al.,* 2018). The workflow and data sources of this study are summarised in Fig. 1.

Biting midge collection and identification

The method of biting midge sampling and identification was described in our earlier study (Möhlmann *et al.,* 2018). In short, three habitat types were defined in which *Culicoides*

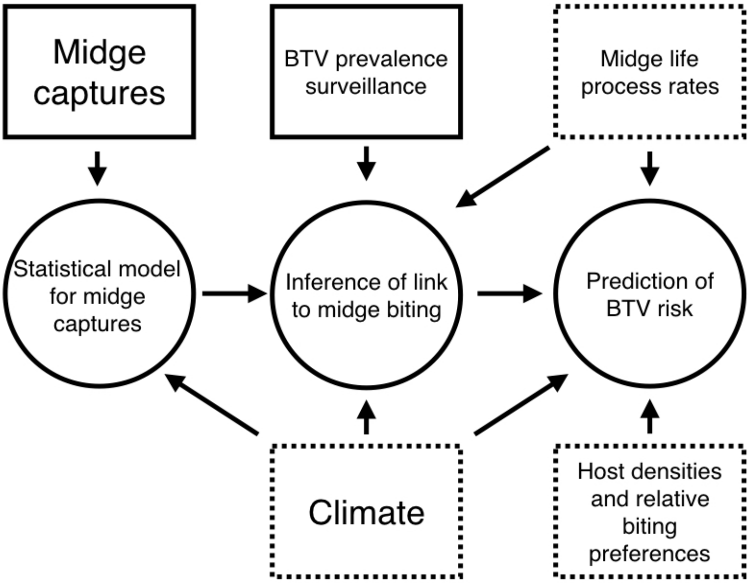

Midge captures
BTV prevalence surveillance
Midge life process rates
Statistical model for midge captures
Inference of link to midge biting
Prediction of BTV risk
Climate
Host densities and relative biting preferences

Figure 1. Schematic overview of the data and modelling workflow. Rectangular boxes represent data sources (solid borders denote data collected for the purpose of this study, dotted borders denote data available from literature or open-access digital archives). Circles represent models developed in this study, which have been either inferred from, or parametrised by, these data sources. Arrows denote dependency in model inference.

specimens were collected: "farm", "peri-urban", and "wetland". Traps were placed within a 50 m radius of cattle, a house, or waterbody, for farm, peri-urban, and wetland habitats respectively. Habitat types generally matched the classification of the CORINE European Land cover database (Heymann, 1994). Collections were performed in Sweden (surroundings of Linköping N58.410808, E15.621532), The Netherlands (surroundings of Wageningen N51.964795, E5.662898), and Italy (surroundings of San Benedetto del Tronto N42.949483, E13.878503). Onderstepoort Veterinary Institute black light traps were placed at three independent locations for each selected habitat type. Traps were at least 100 m apart to prevent overlap of the active trapping radius (Blackwell, 1997). More details and exact trap locations can be found in Vogels *et al.* (2016) and Möhlmann *et al.* (2018). Collections were performed for six consecutive days in each month in all three countries, during the period from July 2014 to June 2015 except the winter months of December, January and February (and March for Sweden). Traps were emptied and repositioned at different locations every 24 hours. Collections were sorted and stored in 70% ethanol at -20°C. Samples were identified to species level using the Interactive Identification Key for *Culicoides* (IIKC) developed by Mathieu *et al.* (2012).

Predictor variables for midge activity

Tinytag® meteorological data loggers (Gemini Data Loggers, Chichester, UK) were used to record local temperature and relative humidity every 30 minutes during the collection period from 17th July 2014 until 3rd July 2015. For each habitat in each country, one data logger was used (3 countries x 3 habitats). Additional meteorological data was collected from a number of sources: hourly wind velocities and local temperatures were available from the weather station closest to the trap location in Sweden (Swedish Meteorological and Hydrological Institute (SMHI) Linköping weather station), The Netherlands (Koninklijk Nederlands Meteorologisch Instituut (KNMI) weather stations "De Bilt", "Deelen", "Cabauw", and "Volkel"), and Italy (San Benedetto del Tronto weather station). Finally, additional minimum, maximum, and mean daily temperatures along with precipitation and air pressure were sourced from the E-OBS European climate database (Haylock *et al.*, 2008). This data was available at a daily temporal resolution and a spatial resolution of 0.25 degrees latitude and longitude. For climatic variables such as wind only one source per area could be used, whereas for temperature we had the opportunity to explore the most predictive of a number of data sources per country. Habitat effect was included by using the habitat types (farm, peri-urban, wetland) as a categorical predictor in the regression models.

Regression model for biting midge catches

A preliminary inference investigation using a hurdle negative binomial model to explain trap catches, inferred using Metropolis-Hastings MCMC, suggested that the excess of zero catches could be explained without zero inflation using the hurdle mechanism. We therefore used GLMM regression for its convenience and flexibility. Regression for the fixed effect coefficients and variance parameters of the random effects was performed via maximum likelihood using the Laplace approximation method implemented by the fitglme MATLAB® function. We followed the recommendation guidelines of Bolker *et al.* (2009) for using generalized linear models in the context of applied ecology, starting from a model with a full set of predictors and performed systematic model reduction using AICc to score model improvement (backwards model selection). AICc is a well-established information criterion for model selection since it is easy to calculate and interpret for GLMMs. The AICc difference between two models (ΔAICc) estimates the relative likelihood of the two models (with $\sim\exp(-\Delta\mathrm{AICc}/2)$ as relative likelihood for the model with greater AICc). Moreover, the model selected by lowest AICc is also the model that would be selected by leave-one-out cross-validation (Stone, 1977).

Candidate predictor variables for removal from the model were chosen by assessing which fixed-effect coefficients had the greatest P value (for the null hypothesis that the coefficient is zero) and which random effect coefficients had the smallest predicted standard deviation. This was followed by trialling the removal of either of the variables and removing the variable that reduced AICc the most, until no further reduction could be made. Several trials were made where we started from a number of different highly over-parameterised models, which all ended with the same best model.

For any given combination of predictor variables, the catches were assumed to be conditionally Poisson distributed, with the conditional mean for each collection defined by the log-link relationship,

$$ln(\mu_{lct}) = \beta \cdot X_{lt} + b_l \cdot Z_{lt} + \rho_{ct} + \varepsilon_{lt}. \quad (1)$$

Where β is the fixed effect regression coefficient that applies to all catches, with X_{lt} denoting the fixed effect predictors at trap location *l* on day *t*. $b_l \sim Normal(0, \Sigma)$ are the location-grouped random effect regression coefficients with covariance matrix Σ, with Z_{lt} denoting the random effect predictors at location *l* on day *t*. The number of midges per catch was highly variable. Therefore, we included an independent random effect for each catch location and day $\varepsilon_{lt} \sim Normal(0, \sigma_\varepsilon)$. This corresponds to assuming that the number of midges per catch is Poisson log-normally distributed (a standard distribution for over-dispersed count data (Elston *et al.*, 2001). Spatial autocorrelation has been found in other midge catch regression studies (Calvete *et al.*, 2008), so we also included an autocorrelation random effect grouped by country of trapping and day; $\rho_{ct} \sim Normal(0, \sigma_\varrho)$. This effect accounts for events influencing trap catch at a wider scale than just the location definition of each trap. See supplementary information for full model variables, regression coefficients and random effect variance estimates as well as AICc improvements from other models.

Midge biting prediction using dynamic transmission modelling

After the initial BTV outbreaks in 2006, the Dutch government decided to establish a sentinel network of cattle herds in the winter of 2006/2007 to monitor the re-emergence of BTV-8 in 2007, using repeated milk ELISA testing (Santman-Berends *et al.*, 2010; Santman-Berends *et al.*, 2013). For study purposes, The Netherlands was divided into 20 compartments based on geographic boundaries as proposed in Commission Decision 2005/393/EC. In each compartment at least 10 randomly selected herds had to be sampled (with at least sixteen cows per herd) to obtain the required sample size. Herds were not necessarily completely BTV-8 seronegative at initial investigation, but cows designated for the sentinel program had to be BTV-8 seronegative at the moment of selection in May 2007. Therefore, dairy herds to act as sentinels for BTV incidence were selected, that had at least sixteen seronegative cows and at least 50 cattle in total. Monthly milk samples were collected from the sentinel cows in each herd unless prevalence had already reached 100%. The first round of monthly testing of sentinel cows was done in June 2007 and continued until January 2008. The monthly milk samples were tested at GD Animal Health, Deventer The Netherlands for antibodies to BTV-8 using a commercially available ELISA test. For further details on the sampling protocol and commercial ELISA see Santman-Berends *et al.* (2010; 2013).

We connected the regression model for daily midge trap catch to a prediction of daily biting on cattle. For this we used a dynamic and mechanistic model of BTV transmission within herds, which was then matched to the data from the Dutch sentinel study. The dynamic BTV transmission model was formulated using disease compartments and rate-based transitions (see Keeling and Rohani (2008) for further details on this class of transmission model). In addition, it is in most respects similar to the model presented by Gubbins *et al.* (2008) in treating infectiousness amongst cattle, and latency amongst midges, as multi-stage processes that evolve deterministically. In particular, we follow Gubbins *et al.* (2008) in modelling the life processes of the infectious and latent midges (mortality, extrinsic incubation of BTV, and biting) as temperature dependent, and therefore varying daily with local background atmospheric temperature. The major difference between the transmission model used in this study compared to previous modelling approaches for BTV is that we model the daily bites per cattle from susceptible midges as changing daily and varying from herd to herd at a rate proportional to the prediction of the midge catch regression model, denoting the proportionality constant ξ (see Fig. 6 for a schematic diagram of the transmission model). We assume that the location-group random effects apply to herds; that is, the biting rate varies among herds and from day to day according to these (unobserved) random effects.

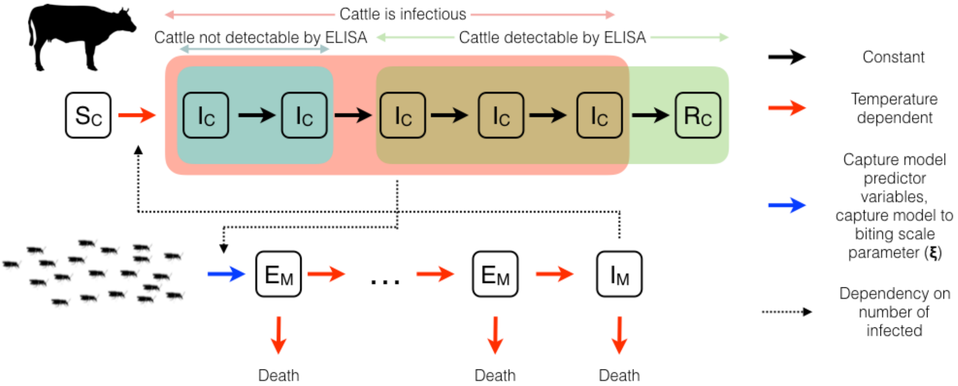

Cattle is infectious
Cattle not detectable by ELISA
Cattle detectable by ELISA
Sc
Ic
Ic
Ic
Ic
Ic
Rc
EM
...
EM
IM
Death
Death
Death
Constant
Temperature dependent
Capture model predictor variables, capture model to biting scale parameter (ξ)
Dependency on number of infected

Figure 6. Schematic representation of the cattle herd level BTV transmission model. The population of cattle and infected biting midges are divided amongst discrete disease compartments. BTV latent midges (E_M) enter the model at a rate proportional to the daily prediction of the catch model. The extrinsic incubation period for latent midges is modelled as a multi-stage process before midges become infectious (I_M). Susceptible cattle (S_C) become infectious cattle (I_C) after a bite from infectious midges (I_M), to become resistant cattle (R_C) in time (also modelled as a multi-stage process; red box). Transitions are shown as solid lines, coloured according to their dependence on environmental variables: constant per-capita (black), daily mean temperature dependent (red), all predictor variables of capture model and the catch-to-bite scale parameter ξ (blue). Dotted lines indicate where the number of infected individuals in one species increases the incidence rate in the other species. Outcomes of the model are linked to observed cattle milk serology time series by the first two infectious stages for cattle with virus being undetectable by ELISA (blue box), whereas subsequent infectious stages and the recovered stage are detectable by ELISA (green box). The likelihood function for ξ was inferred by marginalisation over the latent stochastic variables affecting model outcomes (e.g. herd-specific random effects, daily fluctuations in midge activity). Used images were available under open licence Creative Commons Deed CC0.

In order to infer a proportionality link between the catch model and the daily biting rate (ξ), the outcomes of the dynamic transmission model for each farm were linked to the observed BTV seroprevalence data (see next section). The period during which BTV-infected cattle are detectable using an ELISA test (typically from 8-9 days post-infection onwards (Batten *et al.*, 2008) does not match the period during which the cattle are infectious (rapidly post-infection and then for an average of 20.6 days (Gubbins *et al.*, 2008)). BTV-infected cattle can be in four states that are relevant to transmission modelling and their milk serology: 1) uninfected and susceptible to BTV, 2) infectious but undetectable by milk ELISA, 3) infectious and detectable by milk ELISA or, 4) non-infectious recovered from BTV but still milk ELISA positive. The BTV infectious period for cattle is usually modelled as a 5-stage process (Searle *et al.*, 2013), therefore it was convenient to model cattle in the first two stages of their infectious period (an average duration of 8.2 days) as infectious but undetectable. Cattle in the final three stages of the BTV infectious period are infectious and detectable (see Fig. 6 for a schematic representation of the BTV transmission and serology model).

We inferred a maximum likelihood estimatorfor the trap-to-bite scale parameter by repeated simulation of the percentage of cattle detectable by milk ELISA tests herds in the sentinel herd network. For this we used the climatic conditions of The Netherlands in 2007, and at each simulation repeated redrawing the unobserved random effects for each herd and day. The average likelihood over many repeated simulations corresponds to a Monte Carlo estimate of the true marginal likelihood of the parameter ξ. Estimating the likelihood over a range of values of ξ allowed the construction of a log-likelihood profile.

Inferring the catch-to-biting scale parameter from serological data

The stochastic elements of the piecewise-deterministic BTV transmission model were (i) the herd-grouped random coefficients (this modelled how biting varied from herd-to-herd) and (ii) the daily varying random effects (this modelled how biting varied from day-to-day). It is convenient to denote $W_h = (b_l^{(h)}, \rho_0^{(h)}, \rho_1^{(h)}, \rho_2^{(h)}, \ldots, \epsilon_0^{(h)}, \epsilon_1^{(h)}, \epsilon_2^{(h)}, \ldots)$as the collection of all stochastic elements for herd *h*. For each simulation of the transmission model in each herd, we first drew W_h from their inferred distribution (see supplementary information for distribution parameters of best fitting model).

The likelihood of W_h and ξ for each herd *h* was the chance of selecting the numbers of ELISA seroconverted cattle observed at the herd each month by The Netherlands sentinel study from the underlying distribution of ELISA detectable cattle implied by simulating the transmission model conditional on (ξ, W_h),

$$\mathrm{L}_h(\xi\,,\,W_h) = P\,(\text{Serology data collected at herd } h \mid \xi\,,\,W_h). \qquad (2)$$

Since we were not interested in inferring the specific values of W_h for each herd, they were treated as “nuisance” parameters. We inferred a maximum likelihood estimate, with confidence intervals, for ξ by first estimating the marginal likelihood for ξ (that is the likelihood after integrating over all possible values of the nuisance parameters) at each herd *h*,

$$L_h(\xi) = \int L_h(\xi, w)\, f(w)\, dw. \tag{3}$$

Where *f* is the density function for the distribution of random effects derived from the trapping model. The marginal log-likelihood function for the trap-to-bite scaling parameter, $l(\xi)$, for serological data over a number of herds, was then just the sum of the individual herd marginal log-likelihoods,

$$l(\xi) = \Sigma_h \log L_h(\xi). \tag{4}$$

The herds we chose to contribute to the log-likelihood were those where BTV was found to be already present at the beginning of the study (see supplementary information for more details), to avoid making further assumptions about the introduction mechanism into the herd.

In practice, the log-likelihood was estimated for a profile of values of ξ by simulating multiple realisations of W_h for each herd, that is we estimated (4) by Monte Carlo integration for (3) over a range of values of ξ, and interpolating between points with polynomial regression. The maximum likelihood estimator, ξ^*, was the maximizer of the marginal log-likelihood function presented in the main text along with confidence intervals derived by a standard comparison to the x^2 distribution (see methods section of King *et al.* (2008) for a brief but comprehensive introduction to maximum likelihood estimation using log-likelihood profiles in the context of inference for dynamical systems).

Mechanistic transmission model for BTV transmission within herds

The biting midge catch model allowed us to make a prediction of midge catches on each day and in each regional compartment of The Netherlands whilst the sentinel herd study was ongoing using the E-OBS historic climate records (see above - Predictor variables for midge activity). However, this prediction could not be linked directly to cattle seroprevalence or the true midge biting rate on cattle. To do so, we had to consider the rate at which BTV incubates within midge vectors, the chance of a midge vector transmitting the virus to cattle, and other dynamical factors. These factors were accounted for using a BTV transmission model that took the predictions of the trapping model as an input.

The dynamic and mechanistic BTV transmission model used in this study describes the evolution of the numbers of susceptible, infected, and recovered cattle as well as latent and infectious midges for each herd (Fig. 6). In most respects this model is similar to the model presented by Gubbins *et al.* (2008) in treating infectiousness amongst cattle and latency amongst midges as multi-stage processes that evolve deterministically. Temperature-dependent midge bionomic rates were used for biting frequency of individual infectious midges (Mullens & Holbrook, 1991), the incubation rate of BTV within the midge (Carpenter *et al.,* 2011), and for the midge mortality (Gerry & Mullens, 2000). The midge bionomic rates at each herd on each day were determined by the local mean temperature day according to the E-OBS climate dataset (see above - Predictor variables for midge activity). We modelled the incubation period of BTV within the midge vector as a ten-stage process, which is within the range of best fit models found in meta-analysis and laboratory studies of BTV incubation (Carpenter *et al.,* 2011) (see supplementary information for complete model details and literature estimates for rates).

The major difference between the transmission model used in this study compared to previous modelling approaches is that we model the rate of bites per cattle from the susceptible midges as changing daily and varying from herd to herd,

$$\text{Biting rate of susceptible midges per cattle at herd } h \text{ on day } t = \xi\mu_{ht}. \tag{5}$$

Where μ_{ht} is the expected trap catch given the climate condition local to herd h on day t and ξ is then a scaling parameter between the mean catch prediction and the biting rate prediction. The reason for this approach to modelling the biting from the susceptible midge population was that the background midge population size is unknown at each herd. Therefore, we used trap catch number estimates as a baseline proxy for biting from this unknown sized population of midges. The random effects in the catch model imply that μ_{ht} is a daily varying random variable, and that our transmission model is in the class of piecewise-deterministic Markov processes (Davis, 1984). We assumed that the location-grouped random effects observed in the catch model became herd-grouped random effects for the biting model. In other words, we assumed that the high variance in midge catching between trapping location reflects high variance in midge biting between different cattle herd locations. Although an assumption, this would explain the highly variable intensity of BTV transmission observed between different herds in The Netherlands sentinel survey despite each herd experiencing a similar climate (Santman-Berends *et al.,* 2013). Because the herd locations were known only as geographic compartment occupancy, the daily local climate variables from the gridded E-OBS data used for predicting μ_{ht} were averaged over all spatial grids overlapping the herd's geographic compartment. Accessing detailed and spatio-temporally resolved wind data across Europe was challenging, therefore we used the long-term average wind velocity of The Netherlands weather stations (see above - Predictor variables for biting midge activity) as a constant predictor.

When simulating an outbreak of BTV within a herd, we first determined all relevant climatic predictors for the herd's regional compartment and the daily temperature dependent midge bionomic rates. Second, we generated the herd-grouped and daily varying random effect coefficients which determined how biting at the herd from susceptible midges varied from a median prediction. Third, we solved the resultant deterministic BTV transmission model for each farm using the ode45 MATLAB® function (see Fig. 6 for an overview)

Calculating and mapping the herd reproductive ratio for bluetongue

The reproductive ratio for BTV will differ from day to day and across space. This reflects seasonality and variation in both climatic trends, and the population density of midges and livestock hosts. We approached estimating the reproductive ratio for BTV in the spirit of the case reproductive ratio (Fraser, 2007), using a technique already developed for midges spreading BTV (Brand & Keeling, 2017). That is, we calculated the expected number of secondary cases amongst hosts due to a host initially infected on each day t in each grid cell x whilst taking into account how the conditions for BTV transmission at location x changed after time t, and using the maximum likelihood model for midge biting. The size of each grid cell was determined by the resolution of the relevant climate data. We used the 0.25 degrees longitude and latitude grid resolution of the E-OBS climate dataset to map reproductive ratio estimates for Europe across space and time. More finely resolved data, e.g. cattle and sheep estimates, were represented at this grid scale by taking averages within the coarser grained grid. Cattle and sheep densities across Europe were drawn from the Livestock Geo-Wiki dataset (Robinson *et al.,* 2014).

The average number of secondary BTV cases amongst all hosts (cattle and sheep) given a host initially infected on day t and at grid cell x, is denoted $R^{(C)}(x,t)$ for an initial infected cow and $R^{(S)}(x,t)$ for an initial infected sheep. For both host species, the average number of secondary cases can be calculated by considering; how many days the host's viraemia will last, the rate at which the host is bitten each day, the percentage of the biting midges that will become infected, how many of these biting midges are expected to survive their EIP to become actively infectious, and how many livestock will be successfully infected by those actively infectious midges. The methodology for combining these estimates using information about midge bionomic rates, EIP distribution, and the daily temperatures on each day after the initial host was infected has been developed by Brand and Keeling (2017).

In this study, we adapted the Brand-method for calculating the reproductive ratio to two species, and used the catch-to-biting scalar derived from comparison between the mechanistic transmission model and the herd sentinel serological survey. The cross-transmission between host species depends on how midge bites are distributed between cattle and sheep. We estimated the proportion of midge bites on cattle at grid cell x, $\phi^{(C)}(x)$, given the availability of sheep using a common relative preference model, e.g. Szmaragd *et al.* (2009),

$$\phi^{(C)}(x) = \frac{N^{(C)}(x)}{N^{(C)}(x) + \pi N^{(S)}(x)}. \tag{6}$$

Where $N^{(C)}(x)$ and $N^{(S)}(x)$ are, the local density of cattle and sheep at grid cell x. Parameter π is a measure of the vector preference for sheep compared to cattle; $\pi<1$ indicates preference for cattle, $\pi>1$ preference for sheep. A relative biting study for sheep and cattle has revealed a preference for biting cattle (Elbers & Meiswinkel, 2014), from which we derived an estimate

π=0.115 for use in this study. We combined $R^{(C)}(x, t)$ and $R^{(S)}(x, t)$ into a single reproductive ratio by calculating the leading eigenvalue of the next-generation matrix (Diekmann *et al.*, 1990),

$$R(x,t) = \sqrt{\phi^{(C)}(x)R^{(C)}(x,t) + (1-\phi^{(C)}(x))R^{(S)}(x,t)}. \qquad (7)$$

An attractive feature of using the reproductive ratio as a measure of transmission intensity is its uncomplicated relationship with the persistence of transmission; if $R \leq 1$ then the infectious pathogen cannot persist. However, we expect that the biting rate, and therefore the reproductive ratio, will vary from herd-to-herd. From equations 1 and 5 we see that the rate of biting from the susceptible midge population at each herd on each day depends on the random coefficients, $b_l^{(h)}$, and daily varying random effects, $\rho_{ct}^{(h)}$ and $\varepsilon_t^{(h)}$,

Biting rate of susceptible midges per cattle at herd h on day t (8)

$$\propto exp(b_l^{(h)} \cdot Z_{lt} + \rho_{ct}^{(h)} + \varepsilon_t^{(h)}).$$

The daily varying random effects (ρ and ϵ) are averaged over our estimates for $R^{(C)}$ and $R^{(S)}$ (this can be achieved analytically; see supplementary information for further details), and therefore our estimate of the reproductive ratio does not depend on daily fluctuations in midge activity. However, variation in the herd-grouped random coefficients indicated systematic differences in midge activity between herds that will not 'average out' over time. The distribution of $b_l^{(h)}$ therefore implied a distribution of biting rates for herds within each grid cell on each day, and therefore a distribution of values of R for herds in each grid cell and on each day.

We present the distribution of R for herds by considering the reproductive ratio that would be calculated if the random variable in equation (8), $b_l^{(h)} \cdot Z_{lt}$, took its pth percentile value every day, denoting this reproductive ratio, $R_p(x,t)$. $R_p(x,t)$ estimates the reproductive ratio that p% of herd reproductive ratios are *less than* in grid cell x on day t. Also, we numerically invert the threshold relationship to find the percentage value, $\boldsymbol{P}$, such that $R_p(x,t)$ satisfies the threshold quantity,

$$P(x,t) = \{ 1 - p \in [0,1] \mid R_p(x,t) = 1 \}. \qquad (9)$$

$P(x,t)$ is therefore an estimate of the percentage of herds that could have a multiplying BTV outbreak in grid cell x if BTV was introduced on day t.

To enable spatially extending risk predictions for BTV across Europe certain assumptions about how the midge biting model could be interpolated between the trap locations were necessary. The best regression model for midge catches found significantly different seasonality at the Italian catch locations compared to Sweden and The Netherlands. We assumed that day length was a determinant of midge seasonality and overwintering at different latitudes; for example, the first day of the year with a day length shorter than 9 hours has been associated with the onset of overwintering in UK midges (Searle *et al.,* 2014). We found no midges on days with less than 8.5 hours of daylight when trapping, although catching was not attempted outside of March-November so the number of samples was small. Therefore, at latitudes where no day is ever shorter than 8.5 hours (lower latitudes than 46°N, which is more or less the border of Switzerland and Italy) we used Italian seasonality to predict midge biting. At latitudes that have at least one day shorter than 8 hours (higher latitudes than 49°N) we used the Swedish and Dutch midge seasonality. Between these two latitudes, a linear interpolation between the predictions of the two seasonal models was applied.

All map images were generated using MATLAB® contourf function. Both the livestock density and E-OBS datasets included grid cells that contain only water, these cells were coloured white.

Results

Midge catch data

As presented in Möhlmann *et al.* (2018) (chapter 4) a total of 50,729 *Culicoides* specimens (97.2 % female, 1.3 % male, 1.5 % unidentifiable) were collected across 442 trap catches between July 2014 and July 2015 at a variety of locations (representing farm, wetland and peri-urban habitats) in Sweden, The Netherlands, and Italy using Onderstepoort black light traps (see Methods for further details of the collection protocol). These data were used as input for the modelling analyses presented here. From the collected midges, a total of 45 different species were identified (Möhlmann *et al.,* 2018). The majority of the collections belong to the group of midge species known to vector BTV: the Obsoletus group consisting of *C. chiopterus* (Meigen, 1830)*, C. dewulfi* Goetghebuer, 1936*, C. montanus* Shakirzjanova, 1962*, C. obsoletus s.s.* (Meigen, 1818), and *C. scoticus* Downes & Kettle, 1952 (88.7 %), followed by *C. punctatus* (2.3 %) and *C. pulicaris* (2.2 %). In total 46,697 of the 50,729 caught midges (92%) were known BTV vector species (Table 1). Another known European vector for BTV, *C. imicola*, was not caught during the study of Möhlmann *et al.* (2018) and therefore this species was not included in the analyses. The lowest numbers of midges known to transmit BTV were collected in Sweden (2,964 / 46,697, 6.3%), followed by The Netherlands (10,359 / 46,697, 22.2%) and the highest numbers were found in Italy (33,374 / 46,697, 71.5%). In all countries, abundance was highest in farm habitats. Farm-associated species of *Culicoides* biting midges dominated the catch counts, not only in farm habitats where livestock hosts were present, but also in the other habitat types. This is consistent with findings in other midge catch studies using different trap types (e.g. Rothamsted suction traps (Sanders *et al.,* 2011)).

Table 1. Known midge vectors for BTV. Number of midges trapped for the known vectors of BTV in Europe, for each country (Sweden, The Netherlands, Italy) and habitat type (farms, peri-urban, wetlands). Data derived from Möhlmann *et al.* (2018).

	Sweden			**The Netherlands**			**Italy**			
Midge species	Farms	Peri-urban	Wetlands	Farms	Peri-urban	Wetlands	Farms	Peri-urban	Wetlands	**Total**
Obsoletus group	1760	10	136	7865	43	1448	33069	54	25	44410
C. pulicaris	892	1	6	11	0	18	184	1	0	1113
C. punctatus	120	3	36	191	7	776	38	2	1	1174
Total	2772	14	178	8067	50	2242	33291	57	26	46697

Regression model for trap catches

We used a generalised linear mixed-effects model (GLMM) to explain the aggregated daily catches of all midge species associated with BTV transmission: the Obsoletus group, *C. punctatus,* and *C. pulicaris*. We considered combinations of predictor variables from three groupings of data: (i) climate data, as described in Methods, with mean values and coefficients of variation (CoV) (i.e. the ratio of standard deviation to mean values) over 24 hours, 7 days and 30 days before collection, (ii) habitat categories "farm", "peri-urban", and "wetland", and (iii) a seasonality effect associated with collection time t included by considering annual, bi-annual, and tri-annual periodic sine and cosine functions on t. Predictor variables were associated with particular trap locations and collection times as fixed and/or random effects. We also included interaction terms between habitat and climate variables and between seasonality/habitat and country. See supplementary information for a complete list of model variables considered in the analysis.

Potential predictor variables were eliminated in a stepwise process to find the best regression model for explaining the midge catch data (chosen using lower values of corrected Akaike information criterion (AICc) as the selection criterion; see Methods and supplementary information). The selected climate variables where higher values predicted greater numbers of midges catches were: the mean temperature over 24 hours before catch collection ($P<10^{-7}$) and the mean daily precipitation over the week before catch collection ($P<10^{-3}$). Coefficient of variation for daily precipitation over the 30 days prior to collection was a significant predictor for larger catch size ($P<10^{-10}$), but only as an interaction term with wetland location. Climate variables where higher values predicted lower numbers of midge catches were: the squared mean temperature ($P<10^{-4}$) and mean wind velocity ($P<0.05$) 24 hours before collection. The squared temperature as negative predictor indicated that the activity of the European midge vectors of BTV considered in this paper was maximised at an optimal temperature of 20-21°C (Fig. 2a). Significantly higher counts of caught midges were found at catch locations in farm habitats when compared to wetland or peri-urban habitats (Table 1, $P<10^{-10}$). The midge catch regression model was significantly improved by allowing the seasonal dynamics (driven by sine and cosine functions on time) of midges in Italy to be different from Sweden and The Netherlands. The midge seasonality at the Italian sampling locations could be summarised by using only annual-period predictors, whereas the seasonality observed in Swedish and Dutch traps was more complex involving bi- and tri-annual periodicity (Table S1).

The seasonality in midge catches could not be explained by seasonal variation in temperature alone. The best regression model included periodic predictor variables (sine and cosine functions on *t*) in conjunction with temperature and precipitation effects to predict the seasonality in midge catching (AICc improvement: ΔAICc=102.4 for model without periodic predictor variables). If periodic predictor variables were not included in the set of potential predictors, then the regression model could only predict uni-modal peaks in midge catch abundance, mid-July for Italy and mid-August for Sweden and The Netherlands (Fig. 2b,c; regression predictions smoothed using polynomial regression to investigate trends). However, the best regression model, including both periodic predictor variables and climatic variables, predicts bi-modal peaks in expected catches at the Italian site (peaks at early-May and early-October; Fig. 2b) and an irregular "humped" peak midge catch abundance for the Swedish and Dutch catch locations (peak late September; Fig. 2c). It should be noted that, as trapping was only performed from March to November, we only present statistical fits valid for the period between those months. Besides seasonality, we considered three groups of random effects in our analysis:

1. Particular sampling locations; this random effect group models variation in the regression coefficients between catch locations.
2. A daily varying spatial autocorrelation effect between all catch results in the same country collected on the same day; this random effect models wide-scale but short-term unobserved influences that affect all trap locations in nearby geographic areas.
3. Daily varying overdispersion between individual catches; this accounts for overdispersion in the midge catch distribution compared to the canonical Poisson distribution for count data.

In the best regression model, there are location-group random effects for the intercept (baseline abundance at locations), the response to mean daily temperature, and the response to mean wind velocity over dawn and dusk before collection (see Fig. 3 for a schematic diagram of the catch model and predictions with random effect uncertainty). The location-group random effect on the intercept has a higher inferred variance than the overdispersion effect, indicating that the variability in midge catches between different sampling locations will typically be higher than the daily variability in repeating midge catches at one sampling location. The location-group random effects were negatively correlated amongst themselves, hence we would expect a sampling location with larger than typical baseline abundance to have a smaller than typical response to changes in temperature or wind velocity.

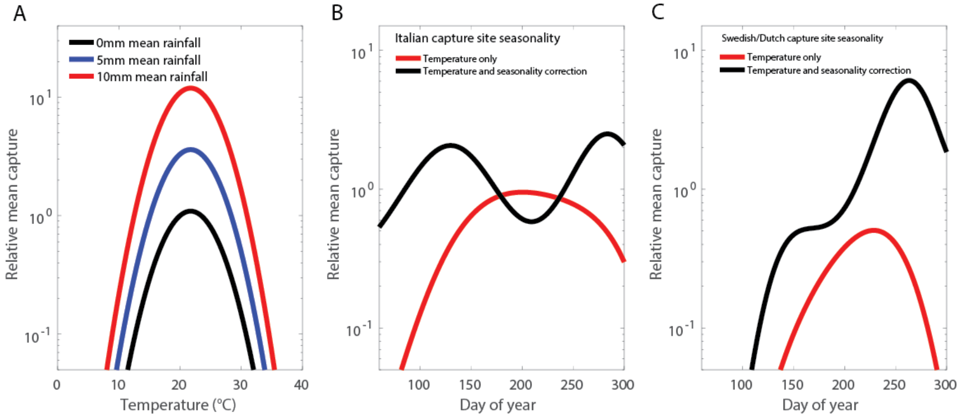


Figure 2. **Expected midge catch size.** Relative effect on midge catch size of varying mean daily temperature, mean precipitation over previous week, and catch day of year (in each case predictor variables being fixed). *Left (A):* Relative expected catch sizes for a range of temperatures and mean precipitation. *Middle (B):* Smoothed seasonal variation at the Italian sampling site if driven by observed local temperatures only (red curve) and with sine and cosine seasonality correction (black curve). *Right (C)*: Smoothed seasonal variation at the Swedish and Dutch sampling sites if driven by observed local temperatures only (red curve) and with sine and cosine seasonality correction (black curve).

Linking midge catch prediction to midge biting prediction

In the winter of 2006/2007, during the BTV transmission-free period, the Dutch government decided to establish a sentinel network of cattle herds to monitor the re-emergence of BTV-8 in 2007 using repeated milk ELISA testing (Santman-Berends *et al.*, 2010; Santman-Berends *et al.*, 2013). Repeated simulation of the percentage of cattle with virus detectable by milk ELISA tests in the sentinel herd network provided us with an inferred maximum likelihood estimator for the trap-to-bite scale. The average likelihood over many repeated simulations corresponded to a Monte Carlo estimate of the true marginal likelihood of the parameter ξ. Estimating the likelihood over a range of values of ξ allowed the construction of a log-likelihood profile.

The maximum likelihood estimator (with a 95% confidence region) derived from the likelihood profile was $\xi^* = 0.53$ [0.40, 0.68] (Fig. S1). This estimator implies that 24 hours of biting midge collection using the Onderstepoort blacklight trap, catches about double the number of biting midges (from the Obsoletus group, *C. pulicaris,* and *C. punctatus)* compared to the number of expected midges to bite a single cow per day.

Bluetongue virus risk in European cattle and sheep livestock herds 2000-2016

We mapped BTV risk in Europe at the resolution of the E-OBS climate data set (0.25° lat./long. grid cells; see Methods). However, the midge biting rate inferred in this study implies

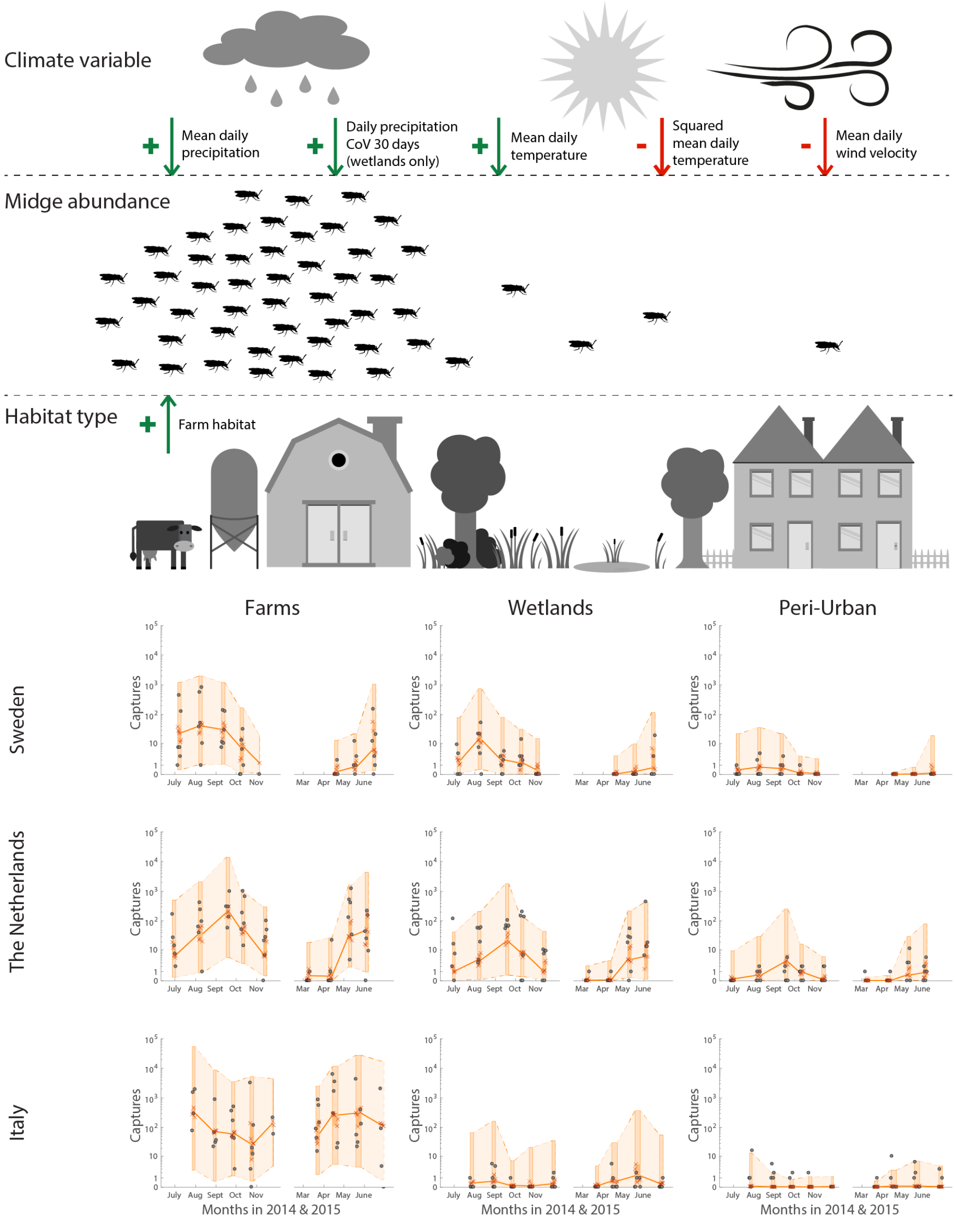


Figure 3. Diagram for midge activity model (top) with midge catch counts and catch model predictions (bottom). Black circles show catch counts and orange crosses are the median prediction of the GLMM over all possible random effects, with orange lines connecting the median predictions of catch weeks. Background shading show GLMM catch predictions between 5%-95% percentiles for random effect coefficients, with deeper shading indicating a collection week. Images created by Viola Visser.

variation between the biting rates of herds, even when they experience the same climate. Therefore, we broadly categorise herds depending on their intrinsic level of risk: "low-risk" herds are bitten at a rate at the 5% percentile of the distribution of biting rates we would expect at their grid cell on each day, "median-risk" herds receive the 50% percentile of biting and "high-risk" herds receive the 95% percentile of biting. We denote the p^{th} percentile herd reproductive ratio R_p. We also calculated, for each day from 2000 to 2016 and each grid cell, the proportion of herds that we expected would have a locally increasing outbreak of BTV upon introduction (P). Where P is the percentage of herds in the grid cell we expect to have a herd reproductive ratio greater than one.

Concentrating first on the grid cells that contained the sampling locations, the Swedish and Dutch sampling locations showed a notable peak in BTV reproductive ratio occurring around late July to early August for each risk level (typical peak reproductive ratio was 3.6 in Sweden and 5.3 in The Netherlands for median-risk herds). In contrast, the Italian sampling locations are predicted to have consistently fairly high BTV reproductive ratios from May to September (typical range was 2.1 - 3.8 for median risk herds) although with less distinct peaks in risk between late May and early September compared to the Dutch and Swedish locations (Fig. 4). At the Swedish sampling locations, median-risk herds are on the borderline for persistence during the early season (May-June; $1< R_{50}< 1.5$) and have only a short period (late July-early August) during which BTV is expected to multiply (i.e. $R_{50} > 1.5$). On the other hand, it was predicted that a high-risk herd in the grid cell containing the Swedish sampling locations would be at risk of a serious outbreak throughout May to August. In the grid cell containing the Dutch sampling sites, high-risk farms will also have a significantly longer transmission season and greatly elevated transmission intensity compared to median-risk herds (Fig. 4a). Each year, the March-November daily mean for R_{50} decreased towards latitudes that are more northern. However, this trend was not observed for peak R_{50}, where peak values for The Netherlands could exceed those in Italy (Fig. 4b). Consistent with other retrospective analyses of BTV risk, we found that 2006 was an outlier year for mean R_{50}.

Our results for p fluctuated daily (see supplementary video 1), therefore we present seasonal averages (Fig. 5). The seasonal average of p for a grid cell can be interpreted as the chance that an infectious animal imported into a local herd in the season, will be introduced into a herd where an outbreak can occur. During the early months of the BTV transmission season (March-May; top row Fig. 5) we found that a large proportion of herds (>75-95%) are already estimated as capable of sustaining BTV outbreaks in a number of regions including: southern Europe (southwest Spain and Portugal, most of mainland Italy), eastern Europe (Hungary, Bulgaria, Romania), southwest France, and the Mediterranean coastal regions of Algeria and Tunisia and western Turkey (Fig. 5). In western Europe (northern France, Germany, and The Netherlands) and in the Baltic States more than half of herds are expected to be already capable of sustaining BTV transmission in these early months. For the most recent five years (2011-2015), there is a notable increase in the percentage of farms capable of sustaining BTV transmission in the Ukraine and Belarus during the early season compared to 2000-2005. This contrasts with findings in Ireland, UK, Denmark, Sweden, where we find that the percentage of herds capable of sustained BTV transmission in March-May was low in the years 2000-2005 (<25-35%) and this has generally remained unchanged by 2011-2015 (Fig. 5).

During the mid-season (June-August) we estimated that BTV transmission could be sustained at more than 80% of herds throughout mainland Europe south of 58ºN, apart from mountainous regions (for example the Alps, Pyrenees, and Carpathians) and in southern/ central Spain. More than 70% of herds were expected to sustain BTV transmission in the midland and southern regions of England and parts of southern Sweden, but we expected

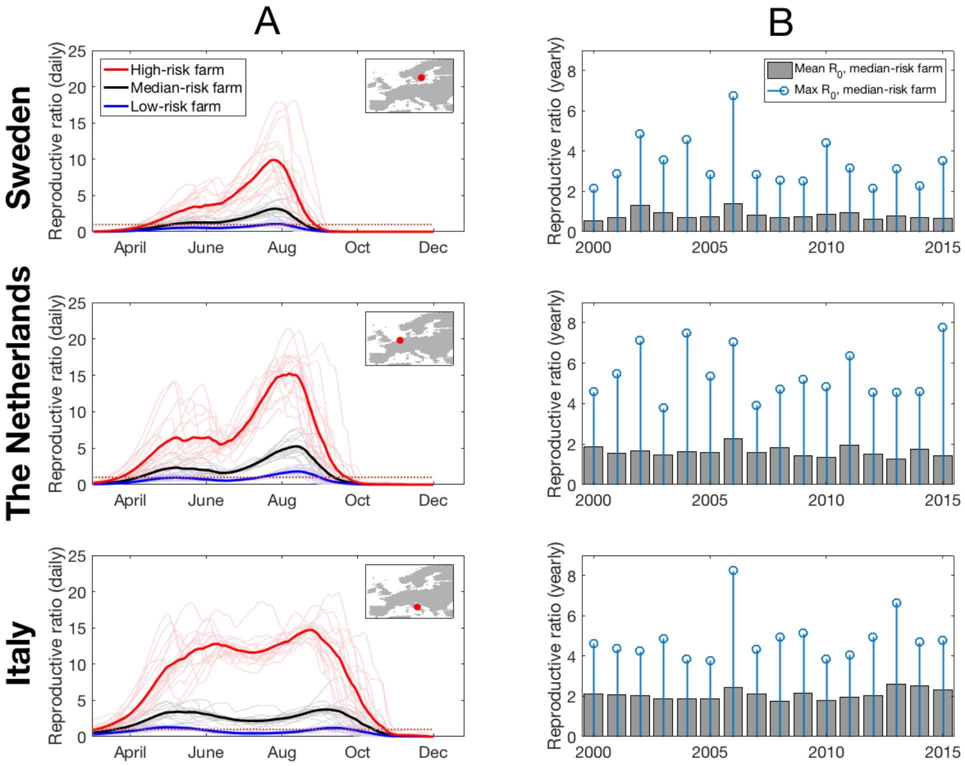


Figure 4. Predicted daily R_p dynamics for farms in the area of the trap sites in each country (shown in insets). Left column (A): R_p on each day of year (March-November) for p=5% (blue curve), median p=50% (black curve) and p=95% farms (red curve) averaged over each year's prediction. Individual years (2000-2015) are shown as fainter curves of same colour. Right column (B): The mean R_{50} (March-November) for each year 2000-2015 (grey bars) with the maximum R_{50} value for each year (blue circles).

lower percentages of herds being capable of sustaining transmission in Norway, Finland, Scotland, Wales, northern Ireland and Ireland. In the last five years considered (2011-2015) the proportion of farms expected to sustain BTV transmission was higher in mainland western Europe (46ºN-53ºN) during the summer compared to 2000-2010. In this most recent period, 95-100% of herds are predicted as being at risk of sustained transmission. In fact, our model predicted that on mainland Europe, BTV risk during the mid-season was higher in the north than further south over the last five years as ideal climatic conditions for BTV transmission (Brand & Keeling, 2017) are more frequently realised. At the tail end of the transmission season (September-November) less than half of the herds across Europe were expected to be able to sustain a BTV outbreak, apart from southwest Spain and Portugal and some coastal regions elsewhere in southern Europe (Fig. 5).

Discussion

By combining multi-scale synthesis of field data and modelling, we connect findings at the scale of individual midge catches in traps to BTV seroprevalence amongst herds of cattle, leading to herd-level risk assessments at the pan-European scale.

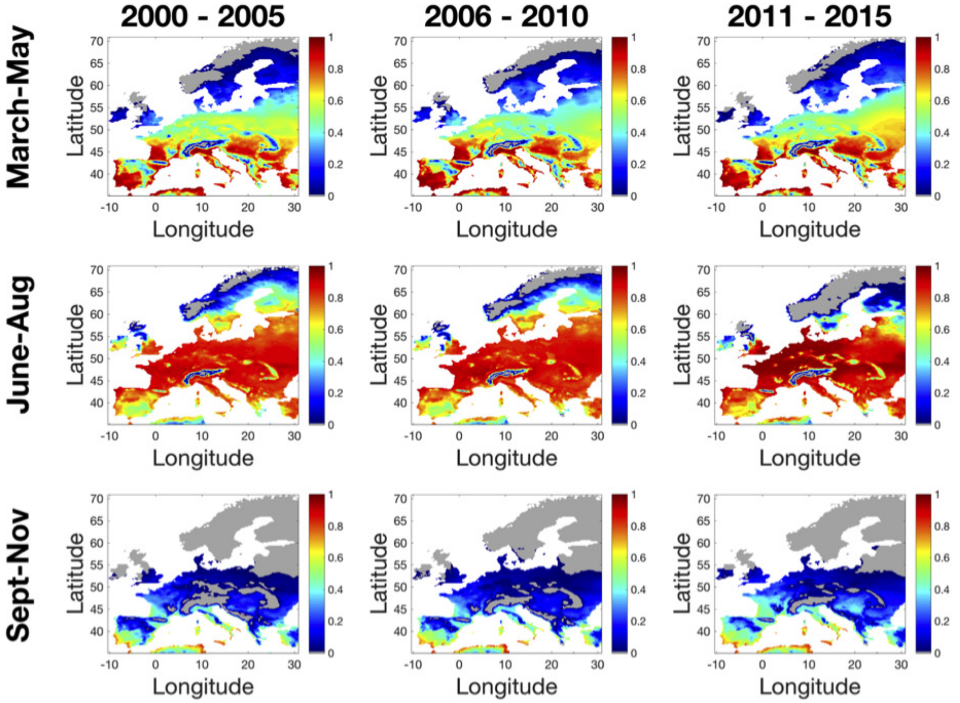


Figure 5. Estimated proportion of naive herds at risk of BTV outbreaks (). Estimations over time periods of five years and early (March-May), mid (June-August) and late (September - November) seasons mapped across Europe for 2000-2015 (spatial cell scale = 0.25º lat./long.). Colours indicate increasing proportion of risk from blue to red (zero risk is coloured grey).

Midge catch studies based on trap catches in a specific country, region, or habitat are reasonably common in the literature. Rather than focusing on midge catches in one country, we spread our trapping effort over a range of different latitudes, thereby observing the activity of BTV vectors under a wider range of climatic conditions. This improves confidence in our regression model, in particular in identifying an optimal temperature for the activity for the BTV vectors found in this study (20-21°C). Trapping studies that focused on warmer southern European countries only, have found that higher temperatures implied a lower probability of catching midges of the Obsoletus group (Calvete *et al.,* 2008). We would conclude that this was due to trapping whilst temperatures were higher than optimal. This makes extending conclusions about BTV transmitted by species from the Obsoletus group based on trapping conducted in a warm climate to north-western Europe unreliable (Guis *et al.,* 2012). On the other hand, in this study we did not find any *C. imicola*, the predominant vector of BTV in southern Europe, most likely because our study sites were outside of the distribution range of *C. imicola* (Calistri *et al.,* 2003). Whilst we extend our predictions to southern Europe for completeness, we acknowledge that our conclusions about BTV risk should be treated with caution in areas where *C. imicola* are abundant, such as large areas of Spain, western Italy, and Mediterranean islands. BTV risk is likely to be underestimated for these regions in our model, as *C. imicola* is thought to be responsible for 90% of the transmission in those areas (Brand & Keeling, 2017). In addition to the identification of an optimal temperature for biting midge activity, we found that larger variation in daily precipitation over the 30 days prior to collection resulted in larger catch size in wetland locations. This precipitation variation effect on midge catches has been detected previously (Calvete *et al.,* 2008). A possible explanation is that higher precipitation variation is correlated with greater availability of unflooded larval development sites (Calvete *et al.*, 2008). Our observation that this effect occurs only in wetland habitats seems to agree with this argument.

A novel feature of the modelling conducted in this study is that it incorporates real field data for both midge catches and transmission to cattle hosts. We were able to infer a proportionality factor between the rate of catching midges and their biting rate from a seroprevalence time series (Mullens *et al.,* 2015), using maximum likelihood for dynamical system inference. This answers a major challenge in the field of vector-borne disease epidemiology by rigorously connecting surveillance to vector biting (Mullens *et al.,* 2015). We found that the biting rate per day was expected to be about 50% of the prediction of 24 hours of trap collection. This is a surprising result in the light of a study where midges were collected by aspiration, that found livestock to be much more attractive to midges than the Onderstepoort black light trap that we used for midge collection in our study (Elbers & Meiswinkel, 2014). However, it should be noted that in the aspiration study, nearly all midges were collected from animals during dusk and collection ceased whilst the rate of trap catches was still rising. The trap-to-biting ratio should be interpreted with some caution since it is inferring the best “effective” biting rate proportional to our catch predictions; that is, the rate of bites that will cause infection. Therefore, errors in estimates drawn from the literature will lead to errors in inference.

For example, if the BTV vector competences used in this study are an underestimate for Dutch midges transmitting BTV-8 then the inferred biting rate to catch ratio will be an overestimate to best fit to the observed cattle BTV-8 prevalence. Better understanding how trap surveillance should inform disease risk cannot be achieved in isolation of improvements in understanding the life processes and vector competence of European midge species as well as the epidemiological differences between the serotypes of BTV. We emphasise that all the serological surveillance data used in this paper was for BTV-8.

We estimate that the risk of BTV outbreaks amongst naive cattle herds has increased in the past five years. South, central, and eastern Europe are estimated to have been at increased risk. Indeed, there has been a succession of outbreaks of various serotypes of BTV between 2010-2015 in both eastern (Hungary, Slovenia, Romania, Croatia) and southern parts (Italy, Spain, southern France) of Europe (Kyriakis *et al.,* 2015), as well as the re-emergence of BTV-8 in central France (Sailleau *et al.,* 2015). High temperatures in southern Europe might decrease BTV risk when conditions become too hot for effective transmission (Brand & Keeling, 2017). However, midge species adapted to these conditions (e.g. *C. imicola*) still pose a threat for BTV transmission in these areas. Moreover, we must emphasise that the reproductive ratio being above unity is a necessary, rather than sufficient, condition for local growth of cases. Therefore, the spatial mapping of BTV risk should be interpreted as the percentage of herds at risk of a multiplying BTV outbreak if BTV was introduced, rather than predicting when and where BTV cases would definitely occur. The actual distribution of cases observed year-on-year also depends upon livestock vaccination coverage, the birth rate of new BTV-naive livestock, and the introduction mechanism of BTV into herds.

In this study, we predict that the reproductive ratio is likely to vary significantly between herds, even if the herds experience similar climatic conditions. We find that the 5% of herds least at-risk, and experiencing climatic conditions identical to those at our trap sites, will virtually never have a strongly growing BTV outbreak. On the other hand, the 5% of herds most at risk could sustain BTV epidemics at any point between March-November if there were sufficient naive cattle. This observation may explain why complete elimination of BTV from European livestock herds remains elusive, despite high vaccination coverage ending significant “travelling wave”-type BTV epidemics, as seen in France in 2007-2009 (Durand *et al.,* 2010). Our prediction that BTV risk will vary strongly from herd to herd is based on statistical regression; we have not identified mechanistic causes of variable risk. A more complete picture of BTV risk requires even more extensive synthesis of midge catches data across Europe, which is an area of on-going research effort (Cuéllar *et al.,* 2018).

Acknowledgements

We would like to thank researchers that helped collecting the data; Rickard Pettersson and Malin Tälle from Theoretical Biology department of Linköping University in Sweden, and Claudia Damiani, Luca Bracchetti and Mahmod Hamdan of the Laboratory of Entomology and Parasitology, University of Camerino in Italy. Without their help, this tremendous amount of field data could not have been obtained. We would like to thank Frans Jacobs for guidance with the identification of the *Culicoides* species. Furthermore, the participating farmers, nature area managers, and homeowners in Sweden, The Netherlands, and Italy that provided us with the essential locations needed for this research are thanked for their cooperation. We acknowledge the E-OBS dataset from the EU-FP6 project ENSEMBLES (http://ensembles-eu.metoffice.com) and the data providers in the ECA&D project (http://www.ecad.eu). We thank Viola Visser for her help with the design of Fig. 3. Finally, we also thank Marcel Dicke for providing comments on a previous version of this manuscript.

Biting midge dynamics and bluetongue transmission: A multiscale model linking catch data with climate and disease outbreaks: Supplementary

Further details on biting midge catch regression

Trap latitude-longitude locations:

		Habitat type		
		Farms	Peri-Urban	Wetlands
Country	Sweden	[58.296530,15.584782] [58.343622,15.602404] [58.330597, 15.704327]	[58.416973, 15.499516] [58.401515, 15.626744] [58.405494, 15.595035]	[58.362106, 15.651861] [58.361585, 15.654910] [58.361542, 15.659072]
	The Netherlands	[51.971084, 5.761455] [51.973637, 5.773978] [52.013077, 5.645998]	[51.979771, 5.660278] [52.018075, 5.655372] [51.979257, 5.645230]	[51.969443, 5.758940] [51.967693, 5.758896] [51.971671, 5.747826]
	Italy	[42.914466, 13.854588] [42.944809, 13.859857] [42.943098, 13.853856]	[42.883455, 13.879388] [42.951012, 13.850783] [42.934424, 13.891933]	[42.896600, 13.911895] [42.899042, 13.909813] [42.903365, 13.908667]

Full list of variables considered for biting midge catch model fitting:

There were three sources of climate data considered for catch model regression: 1) Tinytag® data loggers. One Tinytag® sensor was used for each habitat/country combination and used to represent the climate at all the trap locations of that habitat/country combination. 2) Weather stations. In Sweden and Italy the nearest weather station reporting a time series of climate data was used to represent the climate at all the trap sites in that country. In The Netherlands there were four weather stations approximately equidistant from the trapping locations. The average over all four stations was used to construct the time series of climate data used for all trap sites at the country. 3) E-OBS gridded climate data set (https://www.ecad.eu; Haylock *et al.,* 2008). We identified the E-OBS grid square containing each trap location, and used the time series of climate variables associated with that grid square.

Following is a summary of variables considered for biting midge catch regression by source, variable type (e.g. temperature, humidity) and time scale (e.g. average over day, week before catches etc) or by variable category and their subdivisions:

Climate variables:

- Tinytag® sensor at trap location:
- Hourly temperature (°C)
    - Average over 24 hours before collection
    - Average over last 7 days before collection
    - Average over last 30 days before collection
- Hourly relative humidity (0-100%)
    - Average over 24 hours before collection
- Local weather station data:
- Hourly temperature (°C)
    - Average over 24 hours before collection
    - Average over last 7 days before collection
    - Average over last 30 days before collection
- Hourly relative humidity (0-100%)
    - Average over 24 hours before collection
- Hourly wind velocity (ms^{-1})
    - Average over dawn and dusk before collection
- E-OBS gridded climate data set:
- Daily temperature (°C)
- Daily precipitation (mm)
    - Average over last 7 days before collection
    - Average over last 30 days before collection
    - Average 8-30 days before collection
    - Coefficient of variation over last 30 days (std. dev. of daily precipitation divided by mean)
- Daily air pressure
- Day of collection
    - Yearly periodic sine and cosine functions
    - Bi-annually periodic sine and cosine functions
    - Tri-annually periodic sine and cosine functions

Categorical variables:

- Number of hours of daylight exceeding a threshold
- 10 hours of daylight as threshold
- 9 hours of daylight as threshold
- Country

- Sweden/The Netherlands vs Italy
- Each country
- Habitat
- Farm habitat vs not farm habitat
- Each habitat type

Random effect grouping variables only:

- Unique code for each collection (this models overdispersion in catch sizes relative to Poisson model).
- Unique variable for each day of collection and country pair (this models autocorrelation between catches collected on the same day in the same country).

We also considered product effects of climate variables with specific habitat types to account for some climate variables being important predictors for only particular habitat types.

Details of best fit GLMM model for biting midge catch size:

The target for regression was the aggregate number of Obsoletus group*, C. chiopterus,* Pulicaris group *and C. punctatus* midges captured over 24 hours. As well as identifying potential predictor variables (see above), we also performed GLMM model selection. As described in the main text we used backwards model selection (Bolker *et al.*, 2009), to eliminate potential predictor variables from GLMM models containing large numbers of variables by considering improvement in AICc. The full saturated model contained too many variables to be fitted by the MATLAB® fitglme function (the design matrix for the saturated model is not of full rank), and returned an error. Therefore, we used a selection of highly over parameterised models. The presented best fit model was selected each time by the backwards model selection.

We present the best fit model variable coefficients along with standard errors, a t-statistic for the null hypothesis that the variable coefficient is zero and the p-value implied by this t-statistic (degrees of freedom were 427; Table S1). We also present the estimated covariance matrix of the multivariate normally distributed location grouped random effect variables and estimated variances of the normally distributed autocorrelation and overdispersion effects (Table S2). Finally, the improvement in AICc for the best fit model compared to some selected alternative models are shown (Table S3). All GLMM regression was performed using the MATLAB® fitglme function, with the setting that corresponds to the Laplace approximation approach to estimating fixed effect coefficients and the variance structure of the random effects.

Table S1. Fixed effect regression coefficients for biting midge abundance model.

Fixed Effects			
Variable (dataset)	**Estimate (SE)**	**T-statistic**	***p*-value**
Climatic variables			
Mean daily temperature 24hrs before collection (E-OBS)	1.2687 (0.2304)	5.5057	6.3574e-08
Squared mean daily temperature 24hrs before collection (E-OBS)	-0.0292 (0.007401)	-3.9434	9.3884e-05
Mean daily precipitation 7 days before collection (E-OBS)	0.2391 (0.06895)	3.4682	0.0005771
Precipitation CoV 30 days before collection; wetlands only (E-OBS)	1.0584 (0.1543)	6.8572	2.472e-11
Mean daily wind velocity 24hrs before collection (local weather stations).	-0.2692 (0.13154)	-2.0467	0.04130
Habitat variables			
Farm environment (Italy)	8.2523 (0.8906)	9.2658	9.6007e-19
Farm environment (Sweden and The Netherlands)	4.0519 (0.9078)	4.4634	4.9258e-06
Seasonality variables (Italy)			
Intercept	-16.3872(2.0078)	-8.1617	3.7455e-15
Annual cosine	1.9527 (0.6205)	3.1471	0.0017645
Annual sine	0.8710 (0.2397)	3.6336	0.00031352
Seasonality variables (Sweden and The Netherlands)			
Intercept	-11.5931 (1.9051)	-6.0853	1.8919e-07
Annual cosine	4.1756 (1.1193)	3.7305	0.04768
Annual sine	-0.9064 (0.3411)	-2.6573	0.00031352
Bi-annual cosine	2.105 (0.72941)	2.8859	0.0041003
Bi-annual sine	-0.6901 (0.24171)	-2.8551	0.0045102
Tri-annual cosine	1.9411 (0.45136)	4.3006	2.1138e-05

Table S2. Random effects regression covariance matrix for three levels: Location specific effects, autocorrelation and remaining unexplained overdispersion.

Random effect group	Covariance matrix		
Trapping Location			
	Intercept	**Mean daily temperature**	**Mean daily wind velocity**
Intercept	1.6226	-0.1105	-0.0385
Mean daily temperature	-0.1105	0.0223	-0.1444
Mean daily wind velocity	-0.0385	-0.1444	0.1138
Autocorrelation			
Intercept	0.7540		
Overdispersion			
Intercept	1.3479		

Table S3. Change in AICc from the best fit model for some selected variant models.

Alternate regression model	ΔAICc
CoV of daily precipitation as a predictor for all habitat types, not just wetland.	24.9
Wetland and peri-urban habitats as distinct effects	2.2
One seasonal component for all countries	49.1
Different seasonal component for each country	4.6
Independent location-specific random effects	11.6
No location level random effects	75

Mechanistic transmission model details and likelihood of herd serological data

In this section, we expand upon the description of the mechanistic model for herd-level transmission in the main text.

Transmission model at herd level:

We infer a proportionality factor between the biting rate of midges on cattle and the number of midges we would expect to catch over 24 hours, using the sentinel cattle prevalence data as data for the inference. To do this we construct a mechanistic model, which predicts the dynamics of cattle BTV prevalence given:

(i) the temperature dependent rates of individual biting midge bionomics and virology (biting rate, incubation rate, and mortality rate) drawn from the literature,

(ii) the statistical model for 24 hours of biting midge catches (which depends on temperature and other climatic variables) reinterpreted as being proportional to the biting rate per cattle per day from susceptible midges,

(iii) and the proportionality factor ξ (the target parameter for inference) between the catch model and the daily bites on cattle.

For each herd we introduce state variables: numbers of susceptible cattle (S_c), infectious cattle in their *n*th viraemic stage ($I_C^{(n)}$), recovered/immune cattle (R_C), latent infected midges in their *m*th extrinsic incubation period (EIP) phase (), and infectious midges ($E_M^{(m)}$) (see Fig. 6 in main text for a schematic diagram). The dynamics of BTV transmission in each herd follows deterministic per-capita rates determined by the relevant transmission and recovery events:

- Susceptible cattle are infected, becoming infectious, at a rate proportional to the number of infectious midges, the daily biting rate per midge (α), and the probability of transmission to cattle per bite P_{MC}.
- Infectious cattle remain infectious for a gamma-distributed period with mean duration $1/\gamma$, which corresponds to sub-dividing the cattle infectious period into n_i discrete compartments each with mean duration $1/\gamma n_i$.
- Once an infectious cattle completes all its infectious periods it remains immune to re-infection with BTV.
- Each cattle is bitten by susceptible midges at a rate ξB where B is the expected number of midges that would have been caught according to the statistical trapping model and the proportionality factor ξ is to be inferred. Therefore, the rate at which new midges are infected is proportional to; ξB, the number of infectious cattle, and the probability of transmission to susceptible midges per bite (P_{CM}).
- Infected midges are not immediately infectious. Latent infected midges become infectious after a (gamma distributed) EIP, which corresponds to a n_E sub-divided period, each sub-division being completed at a rate νn_E After becoming infectious, midges remain infectious for the rest of their lives.
- Midges die at a rate μ. Note that not all midges will survive their EIP.

We denote the time series of climate variables selected for the best fit regression model (see above) *C(t)*. Midge bionomic processes are temperature dependent, therefore the atmospheric temperature influences both the rate of susceptible midges arriving to bite cattle, and the rate at which midges which have become infected with BTV incubate the virus experience mortality and make post-infection bites. The temperature at time *t* is denoted *T(t)*. The time variable *t* is continuous, however we treat climate variables as being constant over each day and changing as a daily varying step function. Exact herd locations were redacted. However their containing zone was available for each herd (Santman-Berends *et al.,* 2013). The daily values of *C(t)* and *T(t)* for a herd were the spatial average for that day, over all E-OBS grid cells coinciding with, or contained within, the control zone containing the herd.

Wind velocity is a factor in the best fit regression model, but we found it significantly harder to access detailed spatio-temporal data about wind velocities across Europe. The mean daily wind velocity (at dawn and dusk) during the period of midge catch collections was 3.22ms^{-1}. We used this as a point estimate for each day at each herd, which after fitting the proportionality factor ξ effectively corresponds to increasing the variance of the predictions for biting midge catches (since wind velocity was also a location group random variable).

The best fit biting midge catch regression model includes random effects grouped at the level of location and day of collection. This means that some of the random effects are associated with the herd location, while others are associated with the day. These random effects represent unobserved causes of variation in the midge catch. The location group random effects at herd *h* were modelled as drawn from a distribution of values, $b_h \sim Normal(0,\Sigma)$, where the covariance matrix Σ is given above (Table S2). Note that the location group random effects are only drawn once per simulation since they are associated with the location and do not change in time. The autocorrelation (ρ_{ct}) and overdispersion effects (ε_{ht}) vary daily and are redrawn for each day, as independent normally distributed random variables with mean zero and variance as given above (Table S2). From equation (1) in the main text, the rate of biting from the susceptible midge population at herd *h* on each day *t* is conditional on the random effects;

$$B(t) = \xi \exp(\beta \,.\, X_{ht} + b_h \,.\, Z_{ht} + \rho_{ct} + \varepsilon_{ht}) \qquad \text{(S1)}$$

Where the predictor variables for the fixed effects (X_{ht}) and random effects (Z_{ht}) can be derived each day from the climate time series for the herd, as well as the day of year to include seasonality in midge activity. Here β denotes the fixed effect coefficients for the best fit model (Table S1). Equation (S1) expands equation (2) in the main text.

The description above can be summarised as a random ODE model (Han & Kloeden, 2017) (because of the random effects) for transmission at each herd:

$$
\begin{aligned}
&\dot{S}_C = -\alpha(\mathcal{T}(t))P_{MC}\frac{S_C}{N_C}I_M,\\
&\dot{I}_C^{(1)} = \alpha(\mathcal{T}(t))P_{MC}\frac{S_C}{N_C}I_M - \gamma n_I I_C^{(1)},\\
&\dot{I}_C^{(n)} = \gamma n_I I_C^{(n-1)} - \gamma n_I I_C^{(n)} \qquad n = 2,\dots,n_I,\\
&\dot{R}_C = \gamma n_I I_C^{(n_I)},\\
&\dot{E}_M^{(1)} = \xi P_{CM}B(t)\Big(\sum_{n=1}^{n_I} I_C^{(n)}\Big) - \nu(\mathcal{T}(t))n_E E_M^{(1)} - \mu(\mathcal{T}(t))E_M^{(1)}\\
&\dot{E}_M^{(m)} = \nu(\mathcal{T}(t))n_E E_M^{(m-1)} - \nu(\mathcal{T}(t))n_E E_M^{(m)} - \mu(\mathcal{T}(t))E_M^{(m)} \qquad m = 2,\dots,n_E,\\
&\dot{I}_M = \nu(\mathcal{T}(t))n_E E_M^{(n_E)} - \mu(\mathcal{T}(t))I_M
\end{aligned}
\tag{S2}
$$

$\alpha(T)$, $\nu(T)$, $\mu(T)$ are the midge biting, incubation, and mortality rates expressed as functions of temperature (see Table S4 for functions and parameter estimates). Apart from modelling biting from the susceptible midge population as a random process proportional to the prediction of a GLMM fitted to midge catch data, the model described is identical to Gubbins *et al.* (2008). Simulations of this model were performed in MATLAB® using the ode45 solver function.

Likelihood function for seroprevalence data from The Netherlands sentinel survey

In the main text, we described how repeated simulation was used to marginalise over the random effects and estimate the likelihood function for ξ. We now give further details. The 2007 serological surveillance study in The Netherlands for sentinel cattle herds is fully described in Santman-Berends *et al.* (2010;2013). In summary, 270 herds were recruited into the study from 20 control zones covering the entirety of The Netherlands. Each herd had an initial survey of more than 26 cattle (exact numbers varied). The survey occurred in June 2007 (exact initial survey day varied). In some herds there were already seropositive cattle in the initial survey, however in each case at least 16 initially seronegative cattle were select for future testing. Each month from July to December 2007, the herds were revisited and 16 cattle from the initially seronegative group were selected to test for development of seropositivity. Sensitivity and specificity of the ELISA test for BTV is high (Batten *et al.,* 2008), therefore we simplified our likelihood calculations by treating the test as perfect.

As described in the main text Methods we map the timing of ELISA detectability (8-9 days after infection and for a long period after the end of viraemia (Batten *et al.,* 2008)) onto our multi-compartment transmission model: cattle in the first two stages of the multi-stage cattle BTV-infectiousness period, that is on average the first 8.2 days of infectiousness, have not developed sufficient antibodies to be ELISA detectable. Whereas cattle in subsequent stages of infectiousness were detectable by milk ELISA. Furthermore, we assumed that the study duration was short enough that no cattle in the recovered stage had lost sufficient antibodies to become undetectable.

We initialised simulations for each herd by assuming that the proportion of cattle found to be seropositive in the initial survey at each herd represented the true frequency of seropositivity in the herd. We lacked data on whether the cattle confirmed as seropositive were still infectious. We therefore made the maximum entropy choice that the seropositive cattle were distributed evenly between the ELISA detectable model compartments ($I_C^{(3)}$, $I_C^{(4)}$, $I_C^{(5)}$, R_C). It was highly probable that some cattle that were reported as seronegative were infectious but not detectable. Therefore, we also assumed that there were initially as many cattle in each ELISA undetectable model compartment as each ELISA detectable model compartment. These assumptions implied that for each herd the initial values of detectable model compartments obeyed:

$$\begin{gathered} I_C^{(3)}(0) + I_C^{(4)}(0) + I_C^{(5)}(0) + R_C(0) = N \,.\, SP_{to} \\ I_C^{(1)}(0) = I_C^{(2)}(0) = I_C^{(3)}(0) = I_C^{(4)}(0) + I_C^{(5)}(0) = R_C(0) \end{gathered} \tag{S3}$$

Where SP_{to} is the proportion of cattle found seropositive in the initial survey of the herd (on day t_o) and N is the size of the herd.

In 2007 BTV spread across The Netherlands, broadly travelling from south to north. We avoided making additional assumptions about the mechanism of introduction into herds by restricting our analysis to herds in the south of the country (control zones 15-20 in Santman-Berends *et al.* (2010;2013)), where BTV seropositive cattle were found in the initial herd survey (this reduced the number of herds in the data set from 270 to 60). We also assumed that the number of infected midges present at the beginning of the simulation and arriving over the simulated period were negligible compared to the number infected midges generated by the initially infected cattle (that is every midge compartment was initially zero).

Apart from being identified as initially seronegative, the cattle that was tested each month after the initial survey, were not separated from the rest of the herd. Therefore, they are as likely to become infected with BTV as other cattle. For any set of random effects (the full set of random effects for herd h is denoted W_h; see main text), we can numerically solve the transmission model (S2) for the proportion of cattle who have become seropositive by time t amongst the cattle which were found to be initially seronegative:

$$\mathrm{P}(\mathrm{t}; W_h, \xi) = \frac{I_C^{(3)}(t; W_h, \xi) + I_C^{(4)}(t; W_h, \xi) + I_C^{(5)}(t; W_h, \xi) + R_C(t; W_h, \xi) - N \cdot SP_{t_0}}{N - N \cdot SP_0}. \tag{S4}$$

In which we have explicitly included the dependence on (W_h, ξ) in the solution of model (S2). Care was taken to match the simulation time t to calendar time so that the climate each herd experienced in the simulation matched the data on real climatic conditions in 2007.

The likelihood of the testing data for herd *h*, for a particular set of random effects (W_h), and a given value of the scale factor ξ, is the product over binomial probabilities. The likelihood contribution for the ith test on the herd *h* of detecting n_i seropositive cattle from the $n_{test,i}$ tested cattle on testing day t_i, is the binomial probability of the test. The total likelihood contribution of the herd *h* is:

$$L_h(W_h, \xi) = \prod_i \mathcal{B}in(n_i;\, N_{test,i}, P(t_i;\, W_h, \xi)). \tag{S5}$$

Equation (S5) corresponds to equation (2) in the main text.

As described in the main text the marginal likelihood for ξ without random effects was found by repeatedly simulating model (S2) and using that,

$$\frac{1}{n}\sum_{k=1}^{n} L_h(W_h^{(k)}, \xi) \rightarrow_{n\to\infty} \int L_h(\mathrm{w}, \xi) f(w)\, \mathrm{d}w = L_h(\xi). \tag{S6}$$

Where $W_h^{(k)}$ is the k^{th} independent realisation of the random effects for herd *h* drawn from the density function *f(w)*, which encodes the distribution described by Table S3. For each herd we used n=1000 simulations to marginalise over the random effects for a range $\xi = 0.01, 0.02, \ldots, 1.5$. As described in the main text we constructed a full marginalised log-likelihood for each of these values of ξ by adding the log-likelihoods for each herd. This gave a noisy estimate of the log-likelihood profile $l(\xi)$, which we smoothed using a fourth order polynomial. From the smoothed log-likelihood profile we derived the maximum likelihood estimator $\xi^* = 0.53$ given in the main text. A 95% confidence region $CI(\xi) = [0.40, 0.68]$ was also derived as the region of ξ values with log-likelihood 'close' to the maximum log-likelihood,

$$CI(\xi) = \{\xi \mid 2[l(\xi^*) - l(\xi)] < c\} \tag{S7}$$

The 'closeness' threshold *c* is defined by $\mathbb{P}(\chi_1^2 < c) = 0.95$, this comparison between log-likelihood profile and a chi-squared random variable (with 1 degree of freedom) is a standard method for constructing confidence regions (see King *et al.* (2008) for further description in the context of inference for dynamical systems).

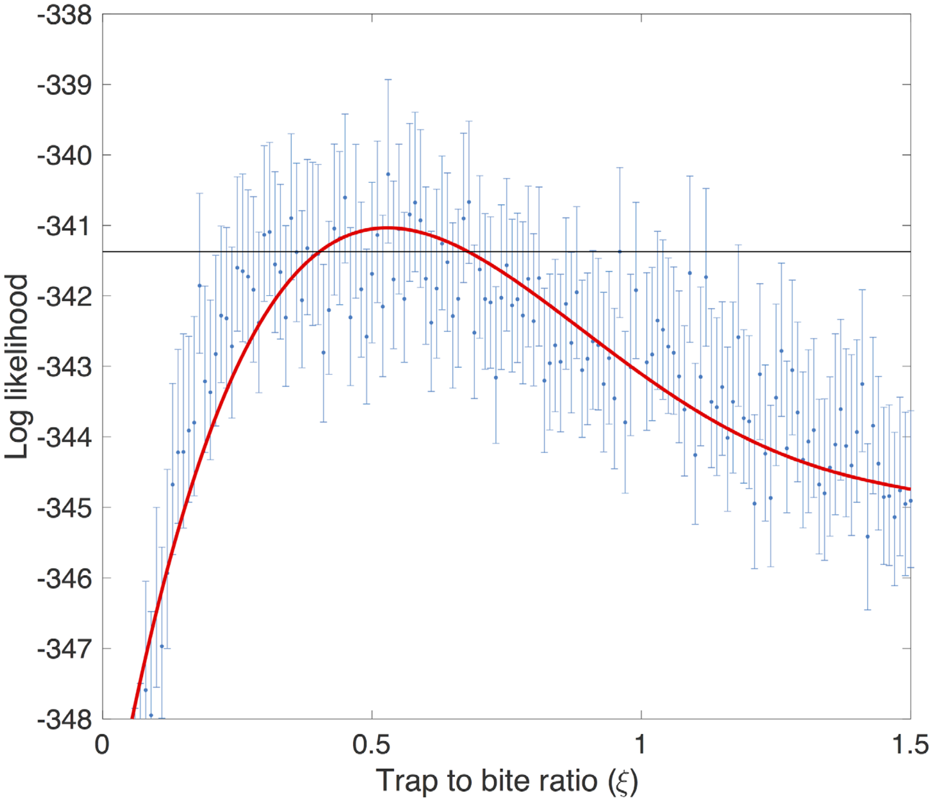


Figure S1. The log-likelihood profile for the trap to bite ratio scalar (ξ). Blue dots and error bars represent the log-likelihood estimate and uncertainty for each value of ξ. The red curve gives a fourth order polynomial smoothing of the noisy log-likelihood estimates from which we calculate a maximum likelihood estimate and 95% confidence region (values of above the black line).

Estimates for epidemiological parameters for herd transmission model

Table S4. Parameters for herd level transmission model. Estimate range for temperature dependent rates are their 10°C - 30°C values.

Description	Symbol	Estimate	Comments	References
Probability of transmission from vector to host	P_{MC}	0.9	Point estimate within range.	Gubbins *et al.*, 2008 Baylis *et al.*, 2008
Probability of transmission from host to vector	P_{CM}	0.05	Point estimate within range.	Carpenter *et al.*, 2006
Midge biting rate	$\alpha(T)$	0.05-0.40 (day^{-1})	Biting rate depends on temperature T: $\alpha(T) = \frac{T(T-3.7)(41.9-T)^{0.37}}{5000}$	Mullens *et al.*, 2004
Midge mortality rate	$\mu(T)$	0.05-1.1 (day^{-1})	Mortality rate depends on temperature T: $\mu(T) = 0.009\exp(0.16T)$	Gerry & Mullens, 2000
Extrinsic incubation period - number of Erlang stages	n_E	10	Point estimate within range.	Carpenter *et al.*, 2011
Extrinsic incubation period - incubation rate	$v(T)$	0-0.3 (day^{-1})	Incubation rate depends on temperature T: $v(T) = 0.018(T-13.4)$	Carpenter *et al.*, 2011
Viraemic duration of cattle - number of Erlang stages	n_I	5	From viraemic duration data fitted to an Erlang (Gamma) distribution.	Melville *et al.*, 1996 Szmaragd *et al.*, 2009

Additional information on spatially varying reproductive ratio calculations

In this section we give additional information on the calculation method used for spatially varying reproductive ratios presented in the main text. We concentrate on the differences between the method used in this paper and the approach used in Brand and Keeling (2017). In this work we consider sheep and cattle, use a regression model for biting derived for this paper, and include the effect of sheep mortality on the estimate of the reproductive ratio.

The idea is to calculate the case reproductive ratio $R(t)$ for BTV in the sense of Fraser (2007), which calculates the expected number of secondary cases generated by an initial host infected at time t. Thereby taking into account that the conditions for transmission might change with time. This approach is computationally challenging because it requires not only information about the climate drivers for BTV at time t but also all times $\tau > t$, noting that midges infected by the host will persist after the end of the individual's infectious period.

This approach to calculating *R(t)* for BTV is fundamentally different from constructing *R* as a function of temperature (Gubbins *et al.*, 2008; Turner *et al*, 2013) which implicitly assumes that temperature will remain constant over the host's infectious period and the remaining lifetimes of the infected midges which survive the end of the host's infectious period.

In Brand and Keeling (2017) a per-vector capacity *C(t)* was defined as the expected number of successful infections due to a single midge alive on day *t*, and calculated using direct summation over expected outcomes on every day $\tau > t$ (see equation (2.5) in Brand and Keeling). The per-vector capacity could be defined either as "alive at the beginning of day *t*" (as in Brand and Keeling) or "alive at end of day *t* having bitten an infected host". The ratio between the two definitions is the probability of the midge biting an infected host and surviving the day. The first definition is more appropriate when considering a fixed sized midge population, the second definition we used for this model since, by assumption, *B(t)* is our estimate of the number midges biting per cattle on day *t*. The expression *B(t)* hides the dependence on random effect coefficients (equation (S1)). Since the autocorrelation and overdispersion effects vary daily, it is natural to average out their effects using,

$$\mathbb{E}[\exp(\rho_{ct} + \varepsilon_{ht})] = \exp([\sigma_\rho^2+\sigma_\varepsilon^2]/2). \tag{S8}$$

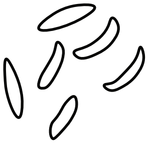
Where σ_ρ^2 and σ_ε^2 are the variances of the autocorrelation and overdispersion effects estimated during the biting midge catch regression (see Table S2 for estimates). We could also average over the location-group random effects, which would lead to an estimate of the reproductive ratio for a 'typical' herd. However, we were more interested in estimating the proportion of farms with reproductive ratio greater than unity across time and space in Europe. Therefore, we used as our estimates of daily bites per animal:

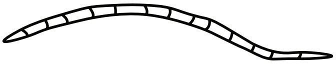
$$\begin{aligned} B_p^{(C)}(t) &= \xi^*\exp(\beta\cdot X_{ht} + [b_h\cdot Z_{ht}]_p + (\sigma_\rho^2+\sigma_\varepsilon^2)/2), \\ B_p^{(S)}(t) &= \pi\xi^*\exp(\beta\cdot X_{ht} + [b_h\cdot Z_{ht}]_p + (\sigma_\rho^2+\sigma_\varepsilon^2)/2). \end{aligned} \tag{S9}$$

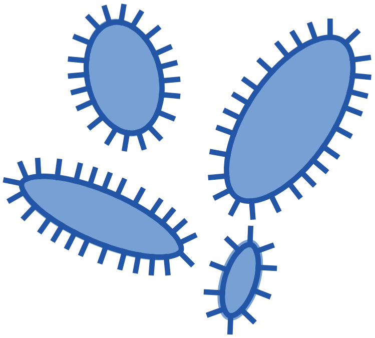
Where $\pi = 0.115$ is an estimate of the relative preference of biting sheep rather than cattle (see main text), and $[b_h . Z_{ht}]_p$ is the p percentile value of the random variable $b_h . Z_{ht}$. Following Brand and Keeling this gives species-specific reproductive ratios at (approximately) p percentile for herds as:

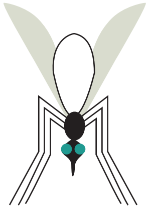
$$\begin{aligned} R_p^{(C)}(t) &= \sum_{\tau=t}^{\infty} B_p^{(C)}(\tau)\hat{C}(t)P_H^{(C)}(\tau,t)\,, \\ R_p^{(S)}(t) &= \sum_{\tau=t}^{\infty} B_p^{(S)}(\tau)\hat{C}(t)P_H^{(S)}(\tau,t)\,. \end{aligned} \tag{S10}$$

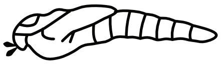
Where $P_H^{(C/S)}(\tau,t)$ are the probabilities that a cattle/sheep infected on day t is still infectious during day $\tau > t$. The infectious duration of cattle is treated as a five stage Erlang process averaging 20.6 days (see above), the time taken to complete a n-stage, mean μ Erlang process (T) is known to be $T \sim \text{Gamma}(n,n/\mu)$. Therefore $P_H^{(C/S)}(\tau,t)= \boldsymbol{P}(T^{(C)}(> \tau - t)$ uses the upper tail function of a $T^{(C)} \sim \text{Gamma}(5,5/20.6)$ random variable. Note that we are assuming that mortality has a negligible effect on cattle, since BTV disease induced mortality was found to be low for cattle during the post-2006 BTV-8 epidemic in northern Europe (Elbers *et al.,* 2008). For sheep excess mortality due to BTV infection is not ignored, we use a point estimate for the per capita disease induced excess mortality rate amongst sheep of $d = 0.0055$ (day^{-1}) within the range of published mortality estimates (Szmaragd *et al.,* 2009; Elbers *et al.,* 2008). The distribution of infectious period for sheep we use has mean 16.4 days: $T^{(S)} \sim \text{Gamma}(14,14/16.4)$ (Szmaragd *et al.,* 2009; Veronesi *et al.,* 2005). Both death and the end of BTV infectiousness stop midge biting on sheep, therefore, $P_H^{(S)}(\tau,t)= \boldsymbol{P}(T^{(S)}(> \tau - t) \exp(-d(\tau-t))$. The factor $\exp(-d(\tau-t))$ is the chance that the infected sheep is still alive $\tau-t$ days after infection. The $R_p(x,t)$ values used in the main text to construct estimates of the percentage of farms in grid cell x that have a reproductive ratio greater than unity on day t were all derived by applying equation (S10) to the main text equation (7).











Chapter 7

Species identity, life history, and geographic distance influence gut bacterial communities in lab-reared and European field-collected *Culicoides* biting midges

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Abstract

Bacteria are part of the insect gut system and influence many physiological traits of their host, such as nutrient availability, development time, longevity and reproduction. Gut bacteria may even reduce or block the transmission of viruses in several species of arthropod vectors. However, only a limited number of studies have investigated the bacterial communities in *Culicoides* biting midges. Knowledge about bacterial communities in different species of biting midges and their related life stages will help to understand how these communities can be manipulated and ultimately used as novel control tools against pathogens. This study assesses how bacterial communities change during development from egg, larva, pupa, to newly emerged and six-day-old adults. To do so, the bacterial communities in the life stages of lab-reared *C. nubeculosus* and *C. sonorensis* were identified using Illumina sequencing of 16S rRNA. In addition, this study investigated how gut bacterial communities are influenced by species identity and geographic distance among biting midge populations. To this end, the gut bacterial communities of five species collected in wetland habitats, and four species collected from farm habitats in Sweden, The Netherlands and Italy were identified. The bacterial community composition of the two lab-reared species significantly changed after pupation and with maturation into six-day-old adults. *Pseudomonas,* Burkholderiaceae and *Leucobacter* bacteria were part of a core community that was transstadially transmitted and found throughout their life cycle. Among field-collected biting midges, the bacterial communities were unique for almost each species. *Cardinium, Rickettsia* and *Wolbachia* were some of the most abundant bacteria found in biting midge species collected from wetlands. Only *Pseudomonas* was present in high relative abundance in all field-collected species. Species identity as well as geographic distance influenced the gut bacterial communities of farm-associated biting midges. These differences in bacterial communities among species and geography might partly explain the observed inter- and intra-species variability in vector competence, whereas stably associated bacteria could be potential new candidates for paratransgenic strategies to control vector-borne pathogens.

Keywords: Illumina sequencing, *Cardinium*, *Wolbachia, Pseudomonas, Asaia*, Obsoletus group, microbiota, life stages, vector, symbionts, Obsoletus complex, 16S rRNA gene

Introduction

Culicoides biting midges are the most important vectors of pathogens that cause animal diseases such as African horse sickness, bluetongue and Schmallenberg. Outbreaks of these diseases have a tremendous impact on livestock welfare and, subsequently, cause considerable economic losses due to animal mortality and trade restrictions (Van Schaik *et al.*, 2008; Saegerman *et al.*, 2014; Veldhuis *et al.*, 2014). Trade restrictions are among the few available options to control disease outbreaks. However, adult biting midges can be dispersed up to hundreds of kilometres with the help of wind, and thereby reduce the effectiveness of trade restrictions (Ducheyne *et al.*, 2007; Hendrickx *et al.*, 2008). Other methods to reduce disease transmission include the control of biting midges by targeting their larval habitats or adult resting sites, the application of repellents or insecticides on host animals, or the housing of livestock in screened (midge-proof) buildings. However, none of these methods is sufficiently effective to drastically reduce biting midge populations (Carpenter *et al.*, 2008; Benelli *et al.*, 2017; Meloni *et al.*, 2018). As an alternative for the reduction of host movement or vector populations, the pathogen itself can be directly targeted to control disease spread. Recent studies show the potential of endosymbiotic bacteria that reduce or block transmission of viruses by arthropod vectors (Hedges *et al.*, 2008; Moreira *et al.*, 2009; Iturbe-Ormaetxe *et al.*, 2011).

Bacterial endosymbionts such as *Wolbachia, Cardinium* and *Rickettsia* are able to influence insect longevity, reproduction and vector competence (Zchori-Fein & Perlman, 2004; Hedges *et al.*, 2008; Iturbe-Ormaetxe *et al.*, 2011; Pilgrim *et al.*, 2017). These factors all influence the vectorial capacity for spread of pathogens. Several studies have shown the presence of *Wolbachia, Cardinium* and *Rickettsia* bacteria in varying proportions for a number of biting midge species (Nakamura *et al.*, 2009; Morag *et al.*, 2012; Lewis *et al.*, 2014; Mee *et al.*, 2015; Pagès *et al.*, 2017; Pilgrim *et al.*, 2017). Recent work showed that the gut microbiota of biting midges can also influence virus infection rates. After manipulation of gut bacterial communities, infection rates with Schmallenberg virus (SBV) of *C. nubeculosus* biting midges were increased (chapter 8). These changes in infection rate were not associated with endosymbionts such as *Wolbachia, Cardinium* or *Rickettsia*. A more elaborate evaluation of the biting midge gut bacterial community is therefore essential. Only a limited number of studies have performed a more comprehensive analysis of the bacterial community in *Culicoides* biting midges. Parker and colleagues (Parker *et al.*, 1977) compared the microbial communities of lab-reared and field-collected *Culicoides* in the USA via culture-dependent methods. Decades later, new techniques were used for analyses of the bacterial communities in *Culicoides sonorensis* and three other biting midge species that are known or suspected vectors of pathogens in the USA (Campbell *et al.*, 2004; Nayduch *et al.*, 2015; Erram, 2016). In Europe, only microbial communities of *C. imicola* have been identified so far (Díaz-Sánchez *et al.*, 2018).

Identification of the gut bacterial community composition is a prerequisite for understanding the functioning of bacteria in their biting midge host. Although earlier work indicates that a change in microbiota has a profound effect on virus infection rates of adult female biting midges (chapter 8), it remains unknown in what stage of their development the adult female acquires its microbiota. If gut bacterial communities can indeed explain variation in infection rates among and within biting midge species (Campbell *et al.*, 2004), insight into the stage of their life cycle in which this community establishes will help to understand how it can be manipulated and, ultimately, how it can be used in novel control strategies targeting the transmission of pathogens.

Gut bacterial communities can have a parental origin as was shown for a selected number of mosquito endosymbionts (Favia *et al.*, 2007; Iturbe-Ormaetxe *et al.*, 2011). However, it was also shown that most of the microbiota present in the mosquito larval stage are removed and excreted during and after metamorphosis into the adult stage (Moll *et al.*, 2001). In addition to vertical transmission of bacteria from the parents, gut bacteria can also be obtained horizontally from the environment. Horizontal transmission may occur through e.g. feeding on nutrients and bacteria in the larval stage, or as adult through uptake of nectar or a blood meal, or via mating (Favia *et al.*, 2007; Wang *et al.*, 2011; Duguma *et al.*, 2015; Díaz-Sánchez *et al.*, 2018). How factors such as environment and diet influence the composition of gut bacteria in vectors remains largely unknown (Erram, 2016; Díaz-Sánchez *et al.*, 2018; Strand, 2018).

We therefore aimed to elucidate in what stage of the biting midge life cycle bacteria become established, and if trans-stadial transmission of gut bacteria occurs. In addition, we aimed to assess to what extent gut bacterial communities differ within and among populations of biting midge species across different geographic distances within Europe.

Materials and Methods

To investigate the origin of gut bacterial communities in adult female biting midges, we selected two lab-reared and nine field-collected biting midge species. The microbiota in all four life stages (eggs, larvae, pupae, adults) of two lab-reared biting midge species (*C. nubeculosus* and *C. sonorensis*) were identified. Gut bacteria of five biting midge species from wetland habitats in The Netherlands were identified. In addition, the gut microbiota of four Obsoletus group species (*C. chiopterus, C. dewulfi, C. obsoletus* s.s. and *C. scoticus*) were determined for biting midges originating from farm habitats in Sweden, The Netherlands and Italy (Fig. 1).

Laboratory-reared biting midges

Two laboratory-reared *Culicoides* biting midge species were used for identification of bacterial communities at different moments in their life cycle. *Culicoides nubeculosus* were provided

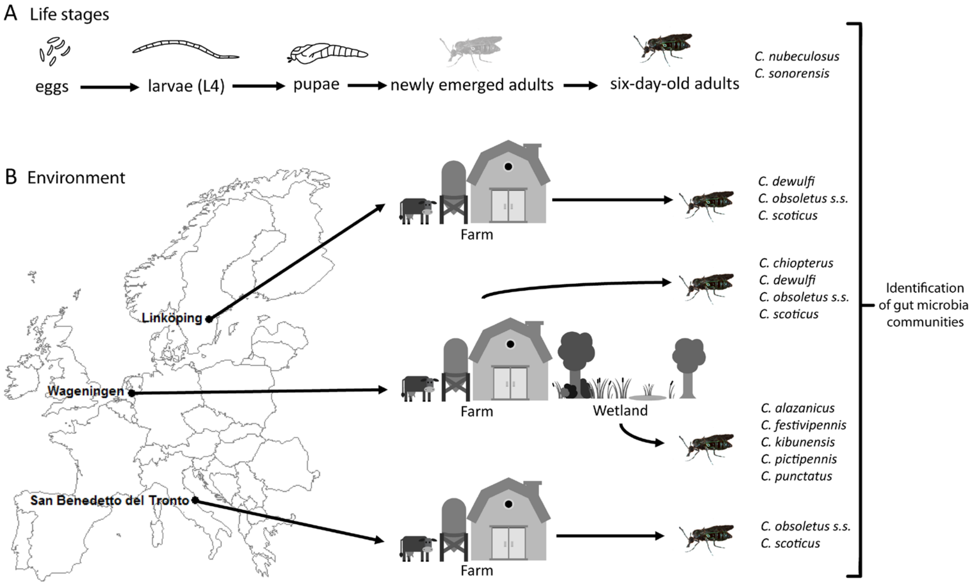

A Life stages
eggs
larvae (L4)
pupae
newly emerged adults
six-day-old adults
C. nubeculosus
C. sonorensis
B Environment
Linköping
Wageningen
San Benedetto del Tronto
Farm
C. dewulfi
C. obsoletus s.s.
C. scoticus
C. chiopterus
C. dewulfi
C. obsoletus s.s.
C. scoticus
Identification of gut microbia communities
Farm
Wetland
C. alazanicus
C. festivipennis
C. kibunensis
C. pictipennis
C. punctatus
Farm
C. obsoletus s.s.
C. scoticus

Figure 1. Overview of biting midge species used for identification of bacterial communities. Panel A. The bacterial composition of eggs, larvae, pupae, newly emerged adults and six-day-old adults of lab-reared *C. nubeculosus* and *C. sonorensis* biting midges were identified. Panel B. In addition, the gut bacterial community composition of female adults of five different biting midge species (*C. alazanicus, C. festivipennis, C. kibunensis, C. pictipennis* and *C. punctatus*) captured in Dutch wetland habitats was identified. Finally, the gut bacterial community composition was identified in Obsoletus group biting midges (*C. chiopterus, C. dewulfi, C. obsoletus* s.s. and *C. scoticus*) from farms in Sweden, The Netherlands and Italy.

by The Pirbright Institute, Pirbright laboratories, United Kingdom (Boorman, 1974), and were maintained at 23±1 °C with 16:8 light:dark cycle and 60% relative humidity. *Culicoides sonorensis* were provided by the Arthropod-Borne Animal Diseases Research Unit, USDA-ARS (Kansas, USA) and were maintained at 25 °C with 16:8 light:dark cycle and 70% relative humidity. Similar rearing protocols were used for both biting midge species (chapters 8 and 9). Briefly, eggs were transferred to trays with filter wool pasted to the bottom (Europet Bernina International, Gemert-Bakel, The Netherlands). Trays were filled with tap water and two drops of Liquifry No.1 (Interpet, Dorking, United Kingdom). Larvae were fed with a 1:1:1 mixture of bovine liver powder (MP biomedicals, Irvine, CA, USA), ground rabbit food (Pets Place, Ede, The Netherlands), and ground koi food (Tetra, Melle, Germany). *Culicoides nubeculosus* larvae were additionally fed with nutrient broth No. 1 (Oxoid, Hampshire, UK). Pupae were transferred to moist emergence cups that were placed in plastic buckets (diameter: 12.2 cm, height: 12.2 cm; Jokey, Wipperfürth, Germany) and closed with netting on the top through which the adult biting midges could feed. Emerged adults were provided with 6% glucose solution *ad libitum*. Bovine blood (Carus, Wageningen, The Netherlands) was provided through a Parafilm M membrane using the Hemotek PS5 feeding system (Discovery Workshops, Lancashire, United Kingdom) for egg production.

Every 2 to 3 days, samples of each life stage from both biting midge species were taken from the rearing in the period from 30 August 2017 to 15 September 2017. Eggs were recovered from filter paper, larvae in the L4 stage and pupae were recovered from larval trays, newly emerged adult females were recovered directly after emergence from pupae, without any exposure to a food source, whereas six-day-old adult females were recovered from a plastic bucket in which the biting midges were provided with 6 % glucose solution *ad libitum*. Each of the selected life stages were surface sterilized by dipping in 70% ethanol for 10 sec, in 5% sodium hypochlorite solution for 60 sec, and finally rinsed in 70% ethanol for 30 sec (chapter 8; Gusmão *et al.*, 2007; Osei-Poku *et al.*, 2012; Erram, 2016). After surface sterilization, at least 500 eggs, five larvae, five pupae or five abdomens of adults were pooled in a 2 ml screw cap microtube (Sarstedt) with a 4 mm borosilicate glass bead (Sigma-Aldrich). For adults, only abdomens were used to include predominantly the gut bacteria. Each sample was replicated 8 times, with the exception of six-day-old adult *C. nubeculosus* (N = 18), eggs of *C. sonorensis* (N = 7) and adult six-day-old *C. sonorensis* (N = 10) (Table 1). All samples were stored in the freezer at -20°C prior to further use.

Field-collected biting midges

Samples of *Culicoides* biting midges were collected in farm, peri-urban, and wetland habitats in three European countries at different latitudes (Sweden, The Netherlands, Italy) as described in (Möhlmann *et al.*, 2018). In short, female biting midges were collected using an Onderstepoort Veterinary Institute blacklight trap and identified to species level using the Interactive Identification Key for *Culicoides* (IIKC) (Mathieu *et al.*, 2010; Mathieu *et al.*, 2012).

Two wetland habitat locations from The Netherlands (wetlands 16 and 18 as described in Vogels *et al.*, 2016 and Möhlmann *et al.*, 2018) were selected, because they had the highest diversity in biting midge species. In total, five biting midge species could be selected from these two locations, because they had been captured in sufficient numbers to create at least six replicate pools of five individuals each. Selected biting midges were dipped in 70% ethanol for 10 sec, in 5% sodium hypochlorite solution for 60 sec, and finally rinsed in 70% ethanol for 30 sec (chapter 8; Gusmão *et al.*, 2007; Osei-Poku *et al.*, 2012). After surface sterilization, the abdomens of five individuals per species were pooled in a 2 ml screw cap micro tube (Sarstedt) with a 4 mm borosilicate glass bead (Sigma-Aldrich). This was replicated six times for *C. alazanicus, C. festivipennis* and *C. punctatus*, whereas seven replicates were prepared for *C. kibunensis* and *C. pictipennis.* This resulted in a total number of 32 pools for the five wetland species (Table 1). All samples were stored in the freezer at -20°C prior to further use.

In addition to the wetland habitats, one farm location was selected for each country (farms 3, 10 and 21 as described in Vogels *et al.* (2016)) for identification of gut microbiota of Obsoletus group biting midge species. A large number of individuals identified as Obsoletus group species was selected for molecular species identification to obtain sufficient individuals per species for the analyses of gut bacterial communities. In total, 554 female Obsoletus group biting midges were identified to species with PCR (as described below). These consisted of 125 individuals from Sweden, 280 individuals from The Netherlands and 149 from Italy.

The selected Obsoletus group biting midges were dipped in 70% ethanol for 10 sec, in 5% sodium hypochlorite solution for 60 sec, and finally rinsed in 70% ethanol for 30 sec (chapter 8; Gusmão *et al.*, 2007; Osei-Poku *et al.*, 2012). After surface sterilization, the abdomen of each individual was removed and stored in a 96 wells plate. The head, thorax and legs were placed in a different 96 wells plate. These parts of the biting midges were used for molecular identification of *C. chiopterus, C. dewulfi, C. obsoletus* s.s. and *C. scoticus* species from the Obsoletus group.

DNA was extracted from the 96 wells plate with head, thorax and legs using a Chelex based extraction method (chapter 5; Miura *et al.*, 2017). First, 30 µL of 5% Chelex® 100 resin (143-2832 BioRad) in ultrapure water was added to each sample in the 96 wells plate. After adding 2 µL 0.5 mg/mL Proteinase K (Ambion), the samples were incubated at 56 °C for 24 hours followed by 3 min at 99.9 °C in a PCR machine. Samples were subsequently centrifuged for 30 seconds at 4,700 rpm before they were used for PCR. For differentiation among species within the Obsoletus group, the protocol as described by Lehmann and colleagues (Lehmann *et al.*, 2012) was used. Ingredients for the mastermix were adjusted for materials used for PCR in our laboratory. For amplification of the cytochrome c oxidase subunit I (COI) region, reverse primer PanCuli-COX1-727R (5'-TATAAACTTCDGGRTGNCCAAARAATC-3') and species-specific forward primers: *C. dewulfi* dew-COI-fwd (5'-CGCCCGACATAGCATTCCCT-3'), *C. obsoletus* s.s. obs-COI-fwd (5'-CAGGAGCTTCTGTAGATTTGGCT-3'), *C. scoticus* sco-COI-fwd (5'-CCACAATTATTAATATGCGATCTACC-3'), and *C. chiopterus* chio-COI-fwd (5'-CCTTTATTTGTTTGGTCTGTTCTTC-3') were used. The mastermix for one sample consisted of 5 µL of 5X colorless reaction buffer (Promega, Wisconsin, USA), 6 µL of $MgCl_2$ (3mM), 5 µL dNTPs (1mM), 2 µL of the forward primer (10µM), 0.5 µL of each reverse primer (10µM), 0.125 µL GoTaq polymerase (5U/µL), 3.375 µL MilliQ, and 3 µL target DNA obtained from DNA extraction. The total volume of 25 µL was used for amplification with PCR settings on 15 min at 94°C, followed by 42 cycles of 30 s at 94°C, 45 s at 63°C, 45 s at 72°C, and a final step of 5 min at 72°C. Final temperature was kept at 4°C until samples were stored in the freezer at -20°C before further use.

PCR products (10 µL) were mixed with Orange G loading dye (5 µL) and loaded on a 1.5% agarose gel for electrophoresis for 45 min at 80 V. A 100 bp ladder was used as reference. A negative control, as well as positive controls for each of the four species, were also included on each of the gels. After electrophoresis the gel was exposed to UV light in a Bio-Rad Gel Doc and imported into the computer program Quantify One to visualise the bands. Species were identified according to differences in PCR product length whereby *C. dewulfi* was 468 bp, *C. obsoletus* s.s. 318 bp, *C. scoticus* 237 bp, and *C. chiopterus* 190 bp (Lehmann *et al.*, 2012).

After identification of the species in the Obsoletus group, five abdomens per species were pooled in a 2 ml screw cap micro tube (Sarstedt) with a 4 mm borosilicate glass bead (Sigma-Aldrich). As expected, the four species of the Obsoletus group were not present in all of the studied countries (chapter 5). Therefore, a total of 20 pools were made for Sweden (1x *C. dewulfi*, 5x *C. obsoletus* s.s., 14x *C. scoticus*), 37 pools for The Netherlands (3x *C. chiopterus*, 12x *C. dewulfi*, 11x *C. obsoletus* s.s., 11x *C. scoticus*) and 18 pools for Italy (9x *C. obsoletus* s.s., 9x *C. scoticus*) (Table 1). This resulted in a total number of 75 pools of Obsoletus group biting midges for the three countries combined that were used for subsequent sequencing of gut bacteria.

Taxonomical identification of gut bacteria

Lab-reared and field-collected biting midge pools were placed in Precellys Evolution tissue homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) and homogenized twice at 7800 rpm for 15 sec. The VWR Mag-Bind Tissue DNA KF 96 Kit (Omega bio-tek, Norcross, GA, United States) was used for DNA extraction of bacterial populations as per the manufacturer's protocol. After extraction, 100 µl was transferred from the elution plate into small Eppendorf tubes and stored at -20 °C until further processing.

Bacterial load was tested for each sample by SYBR Green real-time PCR (Fierer *et al.*, 2005). Five µl of each sample was added to a master mix of 20 µl consisting of 0.12 µl 100 µM Eub338f forward primer, 0.12 µl 100 µM Eub518r µl reverse primer, 10 µl Takara 2x, 0.4 µl ROX2, and 4.36 µl Milli-Q water. The qPCR program was run at 50 °C for 2 min, 95 °C for 10 min, then 40 cycles of 95 °C for 15 sec and 50 °C for 1 min, followed by 95 °C for 15 sec, 50 °C for 1 min, 95 °C for 30 sec and finally 50 °C for 15 sec.

Subsamples of all pools in the dataset were selected for 16S rRNA PCR at different number of cycles as indicated by qPCR. These samples were run on gel to estimate if bacterial DNA yield after PCR was comparable among samples. This process was repeated with adjusted number of PCR cycles until comparable DNA load was achieved. Samples were then placed in triplet in a PCR with 5 µl sample and 20 µl master mix. The master mix consisted of 1.2 µl dNTP (5mM), 6 µl 5xQ5 reaction buffer, 0.15 µl 16S V4 515F forward primer (100 µM), 0.15 µl 16S V4 806R reverse primer (100 µM), 0.3 µl Q5 HF DNA polymerase, and 14.7 µl Milli-Q water (Caporaso *et al.*, 2011)2011. Samples were run in Verity PCR machines with the following program; 98 °C for 30 sec, 98 °C for 10 sec, 50 °C for 30 sec, 72 °C for 30 sec, 72 °C for 2 min and 4 °C until the program was stopped. Number of cycles varied per sample but all were between 16 and 29 cycles. Samples were kept at -20 °C before further processing.

Table 1. Overview of samples used for analysis of the bacterial community composition. Each sample was a pool of at least 500 eggs, five L4 larvae, five pupae or five abdomens of adults. Rearing = Laboratory-reared, Field = Field-collected. NL = The Netherlands, SW = Sweden, IT = Italy.

Origin	Country	Habitat	Species	Life stage	Nr. of samples
Rearing	NL	Rearing	*C. nubeculosus*	Eggs	8
Rearing	NL	Rearing	*C. nubeculosus*	Larvae	8
Rearing	NL	Rearing	*C. nubeculosus*	Pupae	8
Rearing	NL	Rearing	*C. nubeculosus*	Adults newly emerged	8
Rearing	NL	Rearing	*C. nubeculosus*	Adults six-day-old	18
Rearing	NL	Rearing	*C. sonorensis*	Eggs	7
Rearing	NL	Rearing	*C. sonorensis*	Larvae	8
Rearing	NL	Rearing	*C. sonorensis*	Pupae	8
Rearing	NL	Rearing	*C. sonorensis*	Adults newly emerged	8
Rearing	NL	Rearing	*C. sonorensis*	Adults six-day-old	10
Field	NL	Wetland	*C. alazanicus*	Adult	6
Field	NL	Wetland	*C. festivipennis*	Adult	6
Field	NL	Wetland	*C. kibunensis*	Adult	7
Field	NL	Wetland	*C. pictipennis*	Adult	7
Field	NL	Wetland	*C. punctatus*	Adult	6
Field	SW	Farm	*C. dewulfi*	Adult	1
Field	SW	Farm	*C. obsoletus* s.s.	Adult	5
Field	SW	Farm	*C. scoticus*	Adult	14
Field	NL	Farm	*C. chiopterus*	Adult	3
Field	NL	Farm	*C. dewulfi*	Adult	12
Field	NL	Farm	*C. obsoletus* s.s.	Adult	11
Field	NL	Farm	*C. scoticus*	Adult	11
Field	IT	Farm	*C. obsoletus* s.s.	Adult	9
Field	IT	Farm	*C. scoticus*	Adult	9

Sequencing and preparation of data

Samples were sequenced on an Illumina MiSeq platform (Next Generation Sequencing facilities, Wageningen University & Research, Wageningen, The Netherlands). Resulting reads were analysed with QIIME2 (version 2018.8; https://qiime2.org; Caporaso *et al.*, 2010; Bolyen *et al.*, 2018). All forward and reverse reads were demultiplexed and linked to sample-IDs. Sequence-run-specific quality control, merging of forward and reverse reads, removal of 16S V4 primer sequences and of chimeric sequences was performed with the DADA2 package as QIIME2 plugin (Callahan *et al.*, 2016). DADA2 grouped unique sequences equivalent to operational taxonomic unit (OTU) clustering at 100% similarity, resulting in an abundance table of the amplicon sequence variants (ASVs) and a file with the unique sequences. For identification of bacterial sequences we used the amplicon sequence variants (ASVs) instead of molecular operational taxonomic units (OTUs). This has the advantage that data can be more easily re-used and reproduced, and that the obtained ASVs are more closely linked to bacterial species. The advantages of the new ASV approach compared to OTU clustering at 97% similarity have been discussed previously (Callahan *et al.*, 2017)2017. Subsequently, sequences were aligned with MAFFT plugin (Katoh & Standley, 2013) and highly variable positions in alignment were masked (Lane, 1991) to reduce noise in the phylogenetic tree. The FastTree plugin (Price *et al.*, 2010) was used to create an unrooted tree of the unique sequences. The tree was rooted at midpoint of the longest tip-to-tip distance.

Taxonomy was assigned with confidence threshold 0.8 to the unique sequences with Naive Bayes classifier pre-trained on the Silva database (Quast *et al.*, 2012), with release "132 16S V4 region", retrieved from data sources on https://docs.qiime2.org/ with qiime2 classifier plugin (Pedregosa *et al.*, 2011; Bokulich *et al.*, 2018). The ASV abundance table was additionally filtered before further analysis. All sequences were removed that were not classified (Unassigned at Kingdom taxa level), or classified as Eukaryotes, plant mitochondria or chloroplasts, as well as all ASVs without any phylum classification. Very low abundant ASVs with a total count below 10 were also removed as additional noise reduction before further analysis. For analyses performed in R, the QIIME2 data were extracted and abundance tables were converted from BIOM HDF5 to JSON format (McDonald *et al.*, 2012).

Negative control samples

Negative control samples (N = 14) were included that followed the complete protocol from DNA extraction to sequencing. These samples contained no insect material, but generated bacterial sequences nevertheless. Such contaminants can originate from reagents used in the DNA extraction, PCR or next-generation sequencing library preparation, as well as from human skin, oral and respiratory microbiota (Knights *et al.*, 2011; Lazarevic *et al.*, 2016). The 14 samples contained 907 ASVs with a count of 204,153. After filtering of low abundant ASVs as described above, a total of 81 ASVs with a count of 176,225 remained. To identify true contaminants, an occurrence threshold of 20% was used which means that an ASV was present in at least 3 out of the 14 negative control samples. In addition, the selected contaminants together had to contribute 99% to the total fraction counts. A total of 51 ASVs with a count of 140,573 were recognized as true contaminants and filtered from the complete dataset before further analyses. Identified contaminants consisted of several common skin bacteria such as *Corynebacteria*, *Propionibacteria, Staphylococcus* and *Micrococcus* (Grice & Segre, 2011). Together these skin associated ASVs comprised 20% (28,562 / 140,573) of the total counts in the negative controls.

Statistical analysis

The differences in bacterial communities were statistically analysed using a permutation test (999 permutations) based on a redundancy analysis (RDA) of taxa on the treatment factor using Canoco 5.11 (ter Braak & Šmilauer, 2018). All seven taxonomic levels were used simultaneously in these analyses, obtained by summing the ASV counts to the taxon levels kingdom (Bacteria and Archaea), phylum, class, order, family, genus and species. In the analysis, the resulting counts were divided by the library size and the resulting fractions were log-transformed after addition of 0.001, to assist in analyses of the data with many zero counts. The value 0.001 was chosen as its inverse is close to the smallest library size and gives a reasonably symmetric distribution of residuals. The approach has the advantage of yielding one test of significance instead of several level-specific tests. Selection of differentially expressed taxa was based on the percentage fit due to the treatment factor.

For the bacterial community in each sample, alpha diversity indices were calculated for Shannon-Wiener Diversity (H'), the Inverse Simpson Index (D2 or N2), and the Shannon-Wiener Evenness index based N1/N2, where N1= exp(H') and N2 = Inverse Simpson Index using the VEGAN version 2.9.2. package (Oksanen *et al.*, 2009) in the statistical software package R version 3.5.0 (R Development Core Team, 2015, 2017).

Results

Bacterial communities in lab-reared biting midges

To gain insight in the origin of gut bacterial communities in adult female biting midges, the bacterial communities in eggs, larvae, pupae and adults were determined for *C. nubeculosus* and *C. sonorensis*. Two ASVs that belong to the *Pseudomonas* and *Leucobacter* genera were identified in all *C. nubeculosus* life stages (Fig. 2A). Together these two ASVs comprised 11.5% of the total ASV count (3,406,216) in these samples.

With 14 unique ASVs, the pupal stage of *C. nubeculosus* had the highest number of bacterial species that did not appear in high counts in other life stages. Eggs and larvae of *C. nubeculosus* had 7 ASVs in common and bacterial community compositions were comparable between these two life stages (*p*=0.564, Fig. 3A). Larvae and pupae of *C. nubeculosus* shared 10 ASVs; nevertheless, bacterial community compositions were significantly different between these life stages (*p*=0.001). The first principal component (PC), reflecting the difference between the bacterial communities of larvae and pupae, explained 20.5% of the total variance (Fig. 3B). Pupae and newly emerged adults shared 13 ASVs and their bacterial communities were similar (*p*=0.06, Fig. 3C). Finally, newly emerged adults and six-day-old adults shared only 3 ASVs and bacterial communities were significantly different (*p*=0.001, Fig. 3D, the first PC explained 33.2% of the total variation). Six-day-old adults of *C. nubeculosus* did not have any unique ASVs that were not shared with any of the other life stages (Fig. 2A). The bacterial diversity was highest in pupae and newly emerged adults, whereas it was lowest in six-day-old adults (Table S1). In six-day-old *C. nubeculosus* adult females, *Asaia* bacteria were the most abundant with up to 98% of the total bacterial community. These bacteria were found in at least one sample of each life stage. *Asaia* had the lowest prevalence in *C. nubeculosus* eggs and larvae, whereas 50% of the pupal stage samples contained *Asaia* and this increased to 63% and 100% in newly emerged and six-day-old adults respectively. It is unclear how this group of bacteria is picked up, since they are mostly absent in larvae, are not found in the larval habitat of *C. sonorensis* or *C. nubeculosus* (Fig. S1), but are present in approximately half of the pupae.

For *C. sonorensis* also two ASVs were found in all life stages, which were identified as *Pseudomonas* and a species in the Burkholderiaceae. These two ASVs comprised 25.3% of the total ASV count (2,069,107) in the life stage samples of *C. sonorensis* (Fig. 2B). With 12 unique ASVs, the newly emerged adults had the highest number of ASVs that did not appear in high counts in other life stages. Eggs and larvae, larvae and pupae, as well as newly emerged adults and six-day-old adults of *C. sonorensis* only had the *Pseudomonas* and burkholderiaceous ASVs in common. Similar to *C. nubeculosus*, larvae and pupae of *C. sonorensis* had significantly different bacterial communities (p=0.041, Fig. 3F, the first PC explained 13.4% of the total variation). Pupae and newly emerged adults had 19 shared ASVs and had comparable bacterial communities (p=0.084, Fig. 3G). Six-day-old adults of *C. sonorensis* had two unique ASVs (an

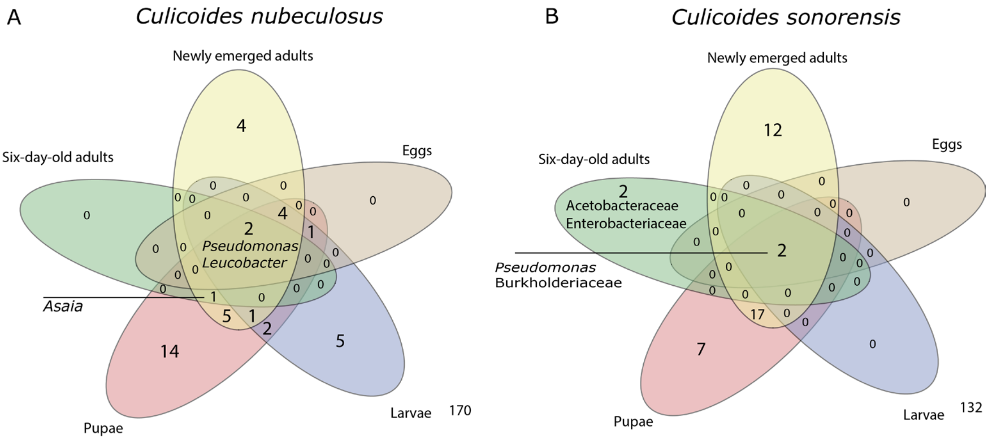


Figure 2. Venn diagrams illustrating overlap in bacterial communities among life stages of *Culicoides* biting midges. Panel A: Number of bacterial ASVs specific and common among *Culicoides nubeculosus* biting midge eggs, larvae (L4), pupae, newly emerged adults and six-day-old adults. Panel B: Number of bacterial ASVs specific and common among *C. sonorensis* biting midge eggs, larvae, pupae, newly emerged adults and six-day-old adults. Names reflect the genera to which the ASVs belong. The number outside the Venn-diagram indicates the number of ASVs that were excluded from the Venn-diagram based on the used thresholds. ASVs were only included in the Venn-diagram if they accounted for at least 0.1% of the total ASV-count in each sample and if they were present in at least 50% of the samples for each group (see Table 1).

enterobacteriaceous and acetobacteraceous species) that were not shared with the other life stages (Fig. 2B), and the older adults were significantly different from newly emerged adults (*p*=0.002, Fig. 3H). Similar to *C. nubeculosus*, the bacterial diversity was highest in pupae and newly emerged adults, whereas it was lowest in six-day-old adults (Table S1).

The two lab-reared species were compared for each life stage to assess how their bacterial communities may vary despite being reared at almost similar conditions. Interestingly, the bacterial community composition in eggs (*p*=0.704) and L4 larvae (*p*=0.240) of *C. nubeculosus* and *C. sonorensis* were similar (Fig. 4A, B). However, bacterial communities of pupae (*p*=0.006), newly emerged adults (*p*=0.001) and six-day-old adults (*p*=0.001) were significantly different between the two species (Fig. 4C, D, E). The first principal component (PC), reflecting the difference between the bacterial communities of pupae, newly emerged adults, and six-day-old adults, explained 16.5%, 19.7%, and 27.2% of the total variance respectively (Fig. 4C, D, E).

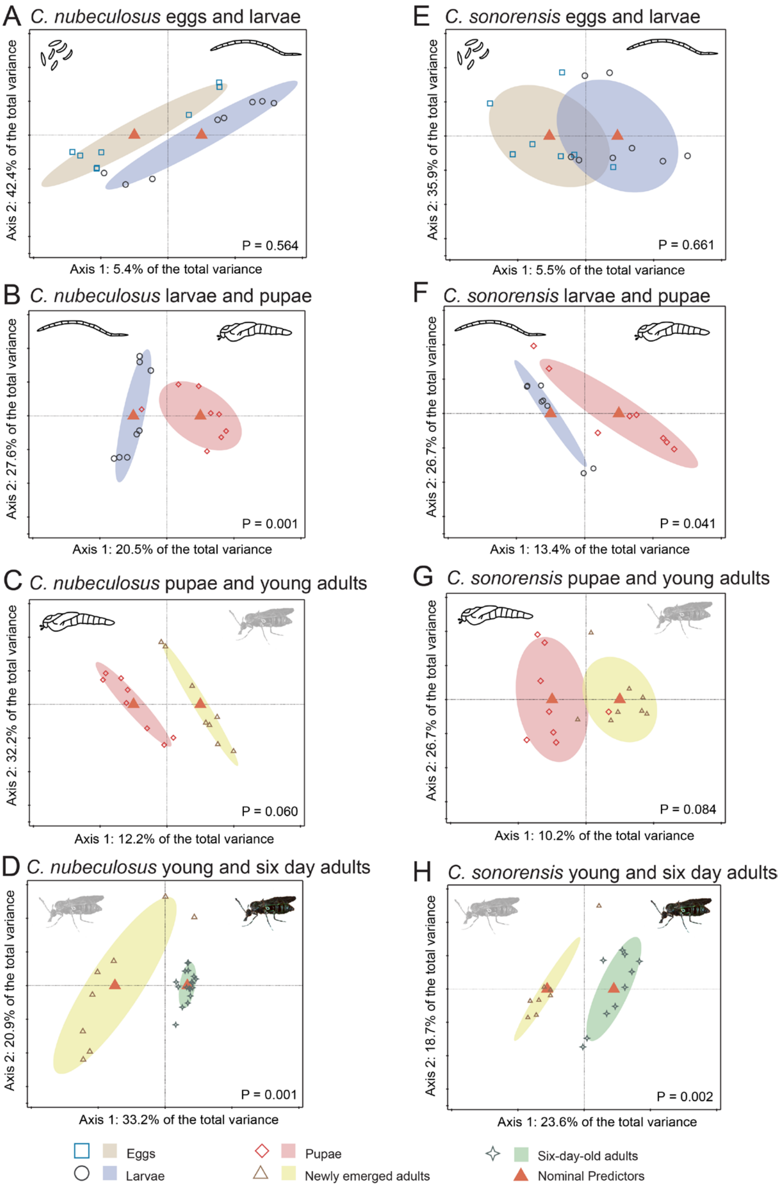


Figure 3. Redundancy analysis (RDA) plots for different life stages of two biting midge species. RDA of logarithm of the fraction of bacteria in *C. nubeculosus* eggs and larvae (Panel A; N = 16, DF = 1, F = 0.8, *p*=0.564), larvae and pupae (Panel B; N = 16, DF = 1, F = 3.6, *p*=0.001), pupae and newly emerged adults (Panel C; N = 16, DF = 1, F = 1.9, *p*=0.06), newly emerged adults and six-day-old adults (Panel D; N = 26, DF = 1, F = 11.9, *p*=0.001) and of *C. sonorensis* eggs and larvae (Panel E; N = 15, DF = 1, F = 0.8, *p*=0.611), larvae and pupae (Panel F; N = 16, DF = 1, F = 2.2, *p*=0.041), pupae and newly emerged adults (Panel G; N = 16, DF = 1, F = 1.6, *p*=0.084), newly emerged adults and six-day-old adults (Panel H; N = 18, DF = 1, F = 5.0, *p*=0.002). Ellipses show 66% confidence levels (±1 time the standard deviation).

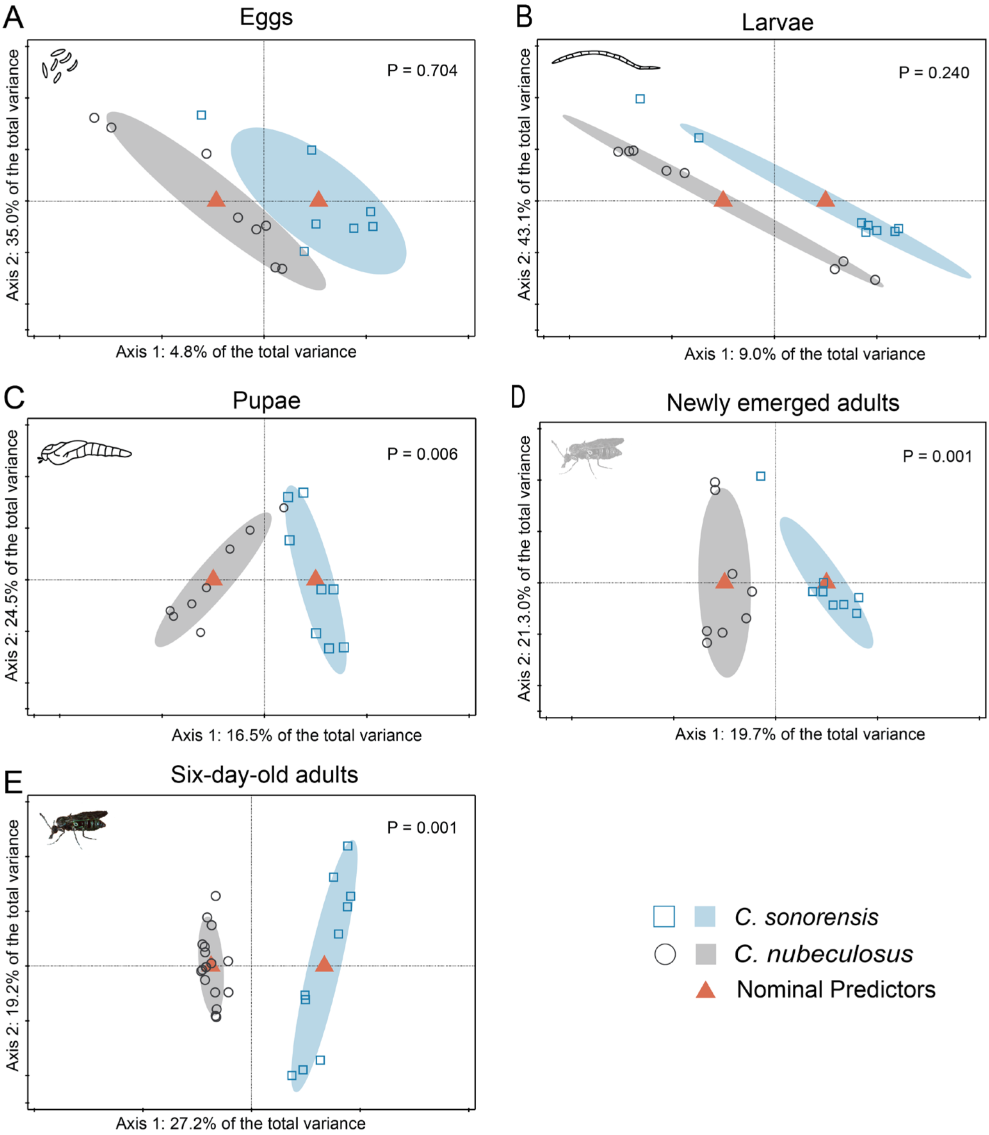


Figure 4. Redundancy analysis (RDA) plots for comparison of bacterial communities of two biting midge species. RDA of logarithm of the fraction of bacteria in *Culicoides nubeculosus* and *C. sonorensis* eggs (Panel A; N = 15, DF = 1, F = 0.7, *p*=0.704), larvae (L4) (Panel B; N = 16, DF = 1, F = 1.4, *p*=0.240), pupae (Panel C; N = 16, DF = 1, F = 2.8, *p*=0.006), newly emerged adults (Panel D; N = 16, DF = 1, F = 3.4, *p*=0.001) and six-day-old adults (Panel E; N = 28, DF = 1, F = 9.7, *p*=0.001). Ellipses show 66% confidence levels (±1 time the standard deviation).

Bacterial communities in field-collected biting midges

To investigate the variation in gut bacterial communities among and within species, nine field-collected *Culicoides* species were selected from a database of 45 species collected in different European localities (chapter 4; Möhlmann *et al.*, 2018). First, gut bacterial communities of five field-collected biting midge species from wetland habitats in The Netherlands were investigated. Second, the gut bacterial community composition of four species from the Obsoletus group were determined for individuals originating from farms in Sweden, The Netherlands and Italy.

Comparing the two lab-reared and the nine field-collected biting midge species revealed that gut bacterial communities of lab-reared individuals were significantly different from gut bacterial communities of field-collected individuals (Fig. 5A, p=0.002). Not a single common ASV could be identified that complied with the thresholds of 0.1% presence in each sample, and presence in at least 50% of the samples within each group. However, a *Pseudomonas* sp. was present in all biting midge species from The Netherlands, with the lowest fraction of 43% presence in samples of *C. pictipennis*, and at least 83% presence in the other species (Fig. 6). Generally, the bacterial diversity was lower in species from wetland habitats (Shannon-Wiener Diversity averages from 0.522 to 1.078 for *C. alazanicus, C. festivipennis, C. kibunensis, C. pictipennis, C. punctatus*) than in species from farm habitats (Shannon-Wiener Diversity averages from 1.216 to 2.253 for *C. chiopterus, C. dewulfi, C. obsoletus* s.s., *C. scoticus*) (Table S1).

The most common ASV in lab-reared *C. nubeculosus,* belongs to the genus *Asaia*. This bacterium occurred in about 50% (43% - 100%) of the field-collected samples, albeit with relatively low counts. The exception was *C. alazanicus,* for which *Asaia* was only identified in one sample, but with a proportion of 32% (Fig. 6). The highest proportion of *Asaia* in samples of field-collected species was found for *C. pictipennis* (up to 84%). From all samples of the nine field-collected species from The Netherlands, 57% (36/63) contained *Asaia* at a level of at least 0.1% of the total ASV count.

The endosymbiont *Rickettsia* was found in field-collected samples of *C. dewulfi* (8% of the samples), *C. obsoletus* s.s. (9%), *C. pictipennis* (14%) *C. scoticus* (18%), *C. kibunensis* (29%) and *C. alazanicus* (67%) as well as in the lab-reared *C. sonorensis* (40% of the samples). In three of the *C. alazanicus* samples *Rickettsia* comprised between 89-91% of the total bacterial community (Fig. 6B, C).

Cardinium was found in field-collected samples of *C. scoticus* (9% of the samples), *C. kibunensis* (14%), *C. alazanicus* (50%), *C. punctatus* (100%) and *C. festivipennis* (100%) as well as in the lab-reared *C. sonorensis* (10%). It was most abundant in five of the *C. punctatus* and five of the *C. festivipennis* samples where *Cardinium* comprised between 30-99% and 43-90% of the total bacterial community for samples of these species respectively (Fig. 6B).

The endosymbiont *Wolbachia* was found in field-collected samples of *C. dewulfi* (8% of the samples), *C. obsoletus* s.s. (18%), *C. scoticus* (18%), *C. chiopterus* (33%) and *C. kibunensis* (86%). No *Wolbachia* was identified in the two lab-reared species. *Wolbachia* was most abundant in six of the *C. kibunensis* samples where this bacteria comprised between 63% and 98% of the total bacterial community identified (Fig. 6B,C).

An anaplasmataceous species was found in field-collected samples of *C. kibunensis* (14% of the samples), *C. chiopterus* (33%), *C. obsoletus* s.s. (36%) and *C. scoticus* (64%) as well as in the lab-reared *C. sonorensis* (30%). It was most abundant in six of the *C. scoticus* samples where the anaplasmataceous species comprised between 66-99.7% of the total bacterial community identified (Fig. 6B, C).

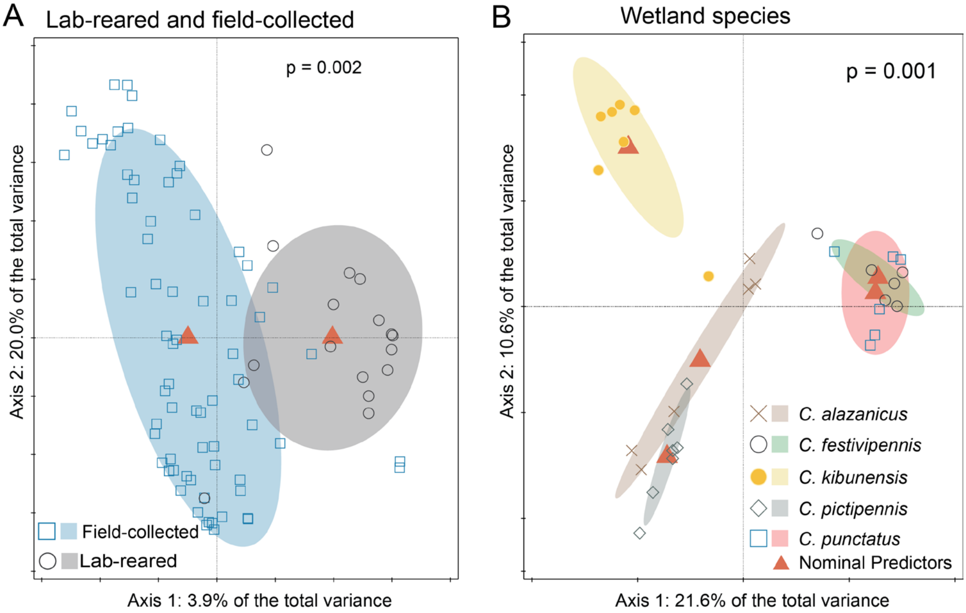
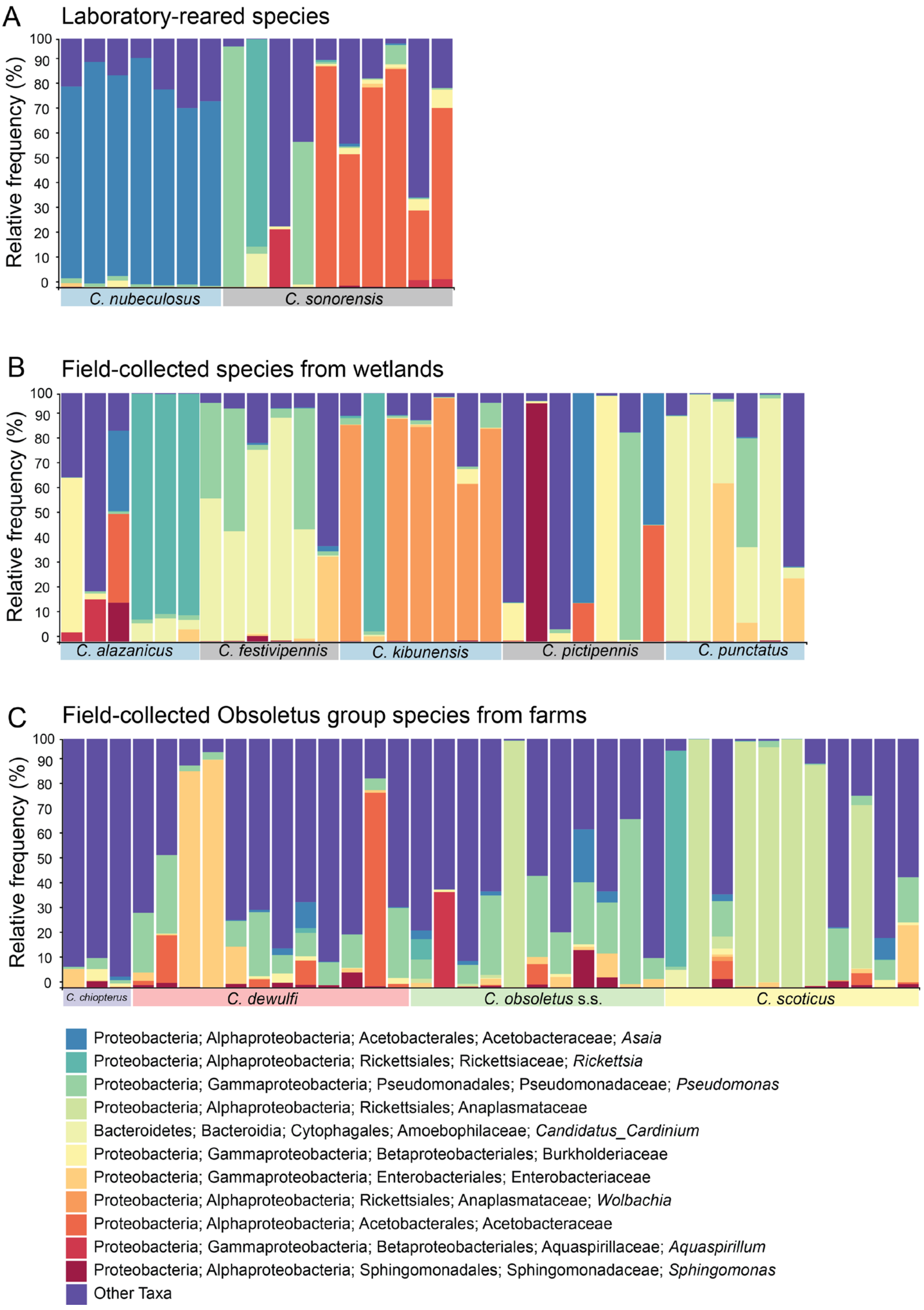


Figure 5. Redundancy analysis (RDA) plots of bacterial communities in laboratory-reared and field-collected female biting midges. Panel A: RDA of logarithm of the fraction of bacteria in lab-reared (*Culicoides nubeculosus* and *C. sonorensis*) and field-collected female biting midges (*C. chiopterus, C. dewulfi, C. obsoletus* s.s. *C. scoticus*, *C. alazanicus, C. festivipennis, C. kibunensis, C. pictipennis* and *C. punctatus*) from The Netherlands (N = 86, DF = 1, F = 3.4, p=0.002). Panel B: RDA of logarithm of the fraction of bacteria in *C. alazanicus, C. festivipennis, C. kibunensis, C. pictipennis* and *C. punctatus* that were collected in wetland habitats in The Netherlands (N = 32, DF = 4.2, F = 3.5, p=0.001). Ellipses show 66% confidence levels (±1 time the standard deviation).


A
Laboratory-reared species
Relative frequency (%)
100
90
80
70
60
50
40
30
20
10
0
C. nubeculosus
C. sonorensis
B
Field-collected species from wetlands
Relative frequency (%)
C. alazanicus
C. festivipennis
C. kibunensis
C. pictipennis
C. punctatus
C
Field-collected Obsoletus group species from farms
Relative frequency (%)
C. chiopterus
C. dewulfi
C. obsoletus s.s.
C. scoticus
Proteobacteria; Alphaproteobacteria; Acetobacterales; Acetobacteraceae; Asaia
Proteobacteria; Alphaproteobacteria; Rickettsiales; Rickettsiaceae; Rickettsia
Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas
Proteobacteria; Alphaproteobacteria; Rickettsiales; Anaplasmataceae
Bacteroidetes; Bacteroidia; Cytophagales; Amoebophilaceae; Candidatus_Cardinium
Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Burkholderiaceae
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae
Proteobacteria; Alphaproteobacteria; Rickettsiales; Anaplasmataceae; Wolbachia
Proteobacteria; Alphaproteobacteria; Acetobacterales; Acetobacteraceae
Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Aquaspirillaceae; Aquaspirillum
Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingomonas
Other Taxa

Bacterial communities in Obsoletus group biting midges

Gut bacterial communities were sequenced for Obsoletus group biting midges originating from farm habitats in Sweden, The Netherlands and Italy. Among the four biting midge species, the gut bacterial composition of *C. chiopterus* was different from the composition in the other three species (Fig. 7A, *p*=0.026).

The gut bacterial communities of *C. scoticus* were significantly different among the three countries (Fig. 7B, *p*=0.007), whereas communities were similar for *C. obsoletus* s.s. from different countries (Fig. 7C, *p*=0.223). Comparing Obsoletus group species within each area we sampled in a country, showed that the gut bacterial communities of *C. dewulfi*, *C. obsoletus* s.s. and *C. scoticus* in Sweden (Fig. 7D, *p*=0.591), and *C. obsoletus* s.s. and *C. scoticus* in Italy (Fig. 7F, *p*=0.093) were not significantly different. However, within The Netherlands, a significant difference among gut bacterial communities of the four species was found, and *C. chiopterus* had the most distinct gut microbial community composition compared to *C. dewulfi, C. obsoletus* s.s. and *C. scoticus* (Fig. 7E, *p*=0.002).

Although *C. chiopterus, C. dewulfi, C. obsoletus* s.s. and *C. scoticus* had different gut bacterial communities, they had four ASVs that were shared among all species (Fig. 7C, H). These bacteria were *Pseudomonas, Bacillus,* an enterobacteriaceous species and *Sphingomonas* which together comprised 28.1% of the total ASV count (1,570,709) in the Obsoletus group samples (Fig. 7H). With 14 unique ASVs, *C. chiopterus* had the highest number of ASVs that did not appear in high counts in the other Obsoletus group species. *Culicoides chiopterus* and *C. dewulfi* had most overlap in bacterial ASVs (17/183), whereas *C. obsoletus* s.s. and *C. scoticus* had the lowest number of shared ASVs (4/183) (Fig. 7H).

← **Figure 6. Abundant bacterial taxa recorded in lab-reared and field-collected female biting midges.** Taxa plots at genus level, presenting the frequency for each taxon, relative to the total number of midgut bacteria in the community composition. The 11 most abundant bacterial taxa are presented for midgut bacterial communities in adult females of the two lab-reared species (*C. nubeculosus* and *C. sonorensis)*, five field-collected wetland species (*C. alazanicus, C. festivipennis, C. kibunensis, C. pictipennis* and *C. punctatus*), and four field-collected farm species (*C. chiopterus, C. dewulfi, C. obsoletus* s.s. *C. scoticus*,) from The Netherlands. Less abundant taxa were grouped as 'Other taxa' to allow a better interpretation of the taxa plots. Each bar represents the relative frequency of bacterial taxa in one pool of five abdomens from a single biting midge species.

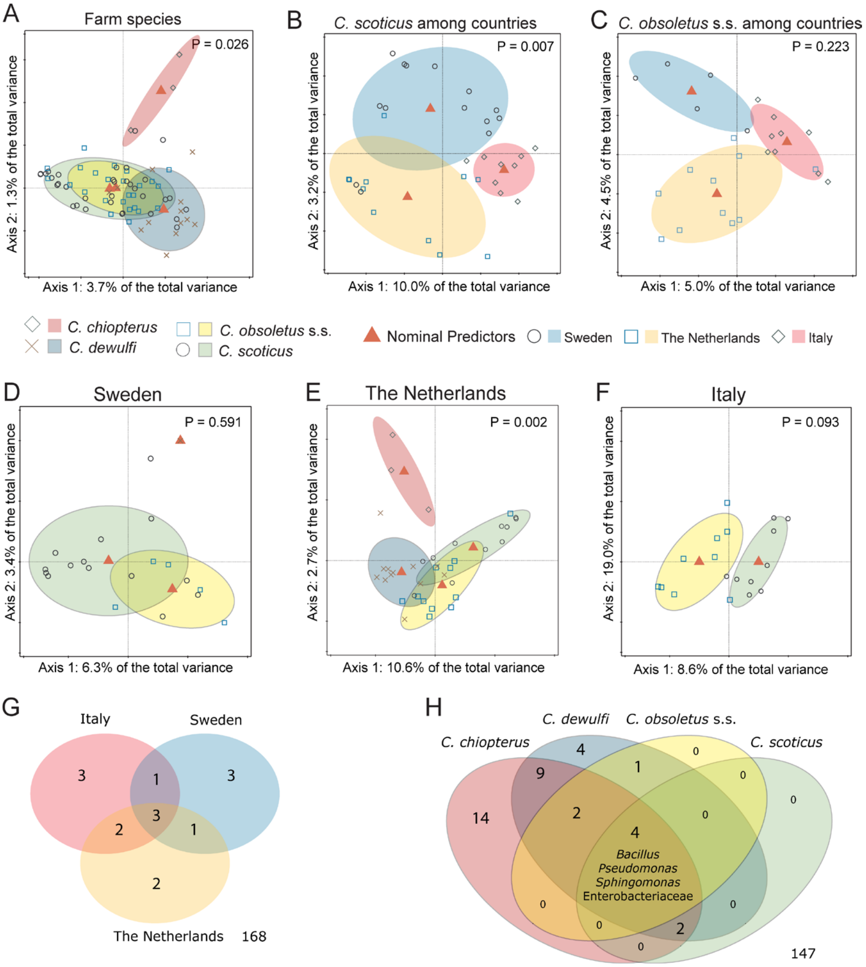


Figure 7. Redundancy analyses (RDA) plots and Venn diagrams for bacterial communities of field-collected Obsoletus group female biting midges. Panel A: RDA of logarithm of the fraction of bacteria in female *C. chiopterus* (diamonds), *C. dewulfi* (crosses), *C. obsoletus* s.s (squares). *C. scoticus* (circles) biting midges originating from three countries (Sweden, The Netherlands, Italy) (N = 75, DF = 3, F = 1.5, *p*=0.026). Panel B: RDA of logarithm of the fraction of bacteria in female *C. scoticus* in Sweden, The Netherlands and Italy (N = 34, DF = 2, F = 2.4, *p*=0.007). Panel C: RDA of logarithm of the fraction of bacteria in female *C. obsoletus* s.s. in Sweden, The Netherlands and Italy (N = 25, DF = 2, F = 1.2, *p*=0.223). Panel D: RDA of logarithm of the fraction of bacteria in female *C. dewulfi, C. obsoletus* s.s. and *C. scoticus* biting midges in Sweden (N = 20, DF = 2, F = 0.9, *p*=0.591). Panel E: RDA of logarithm of the fraction of bacteria in female *C. chiopterus, C. dewulfi, C. obsoletus* s.s. and *C. scoticus* biting midges in The Netherlands (N = 37, DF = 3, F = 2.0, *p*=0.002). Panel F: RDA of logarithm of the fraction of bacteria in female *C. obsoletus s.s* and *C. scoticus* biting midges in Italy (N = 18, DF = 1, F = 1.5, *p*=0.093). Ellipses show 66% confidence levels (±1 time the standard deviation). Panel G: Venn diagram illustrating specific and common bacterial ASVs between Obsoletus group species in Sweden, The Netherlands and Italy. Panel H: Venn diagram illustrating specific and common bacterial ASVs between female *C. chiopterus, C. dewulfi, C. obsoletus* s.s. and *C. scoticus* biting midges originating from Sweden, The Netherlands and Italy. Names reflect the genera to which the ASVs belong. The number outside the Venn-diagram indicates the number of ASVs that was excluded based on the thresholds. ASVs were only included in the Venn-diagram if they accounted for at least 0.1% of the total ASV count in each sample, and if they were present in at least 50% of the samples for each group.

Discussion

Identification of bacterial communities in different life stages of two lab-reared species showed that there are bacterial species that are present throughout their entire life cycle. However, even though the two species were reared under comparable conditions, gut bacterial communities of both pupae and adults differed between the two species. Most of the field-collected species contained unique gut bacterial communities among species from the same environment. Although species identity partially explains differences among gut bacterial communities, we also found an effect of geographical distance on the gut bacterial communities of Obsoletus complex species. We conclude that some bacteria are closely associated with biting midge species throughout their life cycle, as well as with biting midges in general. However, as adults most biting midge species have distinct gut bacterial communities, which might be related to their genetic background as well as environmental factors.

Bacterial communities in lab-reared biting midges

The bacterial communities of different stages in the life cycle of *C. nubeculosus* and *C. sonorensis* were identified to unravel how life stages correlate with their associated bacteria. We show that the process of pupation drastically changes the bacterial community for two lab-reared species. Previous literature showed that gut microbiota in mosquitoes are cleared after moulting both from larval to pupal stage, and from pupal to adult stage (Demaio *et al.*, 1996; Moll *et al.*, 2001). In our study, pupae and newly emerged adults had a similar bacterial community composition for both biting midge species. However, the gut bacterial community of newly emerged adults and six-day-old adults was different. This suggests that the bacterial community composition changes during maturation and after (sugar) feeding of adults. Once adults start to feed on sugar-rich food sources, specific bacteria can utilize these resources, flourish and become largely abundant, which results in a lower bacterial species diversity in their midgut, as was the case for the six-day-old biting midges.

Despite the dynamics in bacterial communities during biting midge development, a selected number of bacterial species was found to occur throughout their life cycle. This shows that some species of bacteria are able to persist during each life stage, including pupation. The same ASV from the *Pseudomonas* genus was present in all life stages of *C. nubeculosus* and *C. sonorensis*. Species in the genus *Pseudomonas* are associated with water and humid environments and they were previously found to be common in biting midge breeding sites as well as in their gut microbiota (Parker *et al.*, 1977; Campbell *et al.*, 2004; Nayduch *et al.*, 2015; Erram, 2016; Díaz-Sánchez *et al.*, 2018). *Pseudomonas* bacteria can provide benefits to insects through protection of eggs against other bacteria, detoxification of polluted larval habitats, promotion of insect growth and facilitation of blood digestion through reduction of oxidative stress after a blood meal (Peck & Walton, 2006; Wang *et al.*, 2011; Alvarez *et al.*, 2012; Senderovich & Halpern, 2013; Chavshin *et al.*, 2015). It is therefore not surprising that these bacteria are closely associated with different life stages of biting midges. Although bacteria from Burkholderiaceae and *Leucobacter* were also associated with all life stages of biting midges in our study, these bacteria seem to be specific for our laboratory rearing environment, as they have only been found in one sample in other studies on biting midge microbial communities (Erram, 2016).

The bacterial communities in eggs and larvae were similar for the two biting midge species that were reared under the same conditions. This indicates that at the start of the life cycle their environment defines the microbiota. Both species were kept in comparable larval rearing conditions and most likely picked up their bacterial community from the surrounding environment, since both *Pseudomonas* and Burkholderiaceae bacteria were also present in larval rearing water (Fig. S1). Similarly, earlier studies showed that diet influences the gut microbiota in insects (Wang *et al.*, 2011; Colman *et al.*, 2012; Yun *et al.*, 2014). In contrast, the bacterial communities of pupae and adults were different between the two lab-reared species. At this point, species identity seems to determine what bacterial species survive after pupation. Our findings support the hypothesis that bacteria can form species-specific associations with certain biting midge species (Erram, 2016).

Because *Asaia* was not present in the larval habitat of *C. nubeculosus*, in low frequency in eggs and larvae, but in more than 50% in pupae and newly emerged *C. nubeculosus* adults, we expect that *Asaia* is transmitted trans-stadially in very low abundance, and only proliferates when conditions become favourable. Adults of *C. sonorensis* had two unique ASVs (Enterobacteriaceae and Acetobacteraceae) that did not occur in high counts in the other life stages and are likely acquired from the sugar solution that is provided as adult food source.

For mosquitoes it is known that bacteria can be acquired from the mothers' genitalia, larval and pupal breeding sites, trans-stadial transmission throughout the life cycle or as adult when feeding on different substrates (Favia *et al.*, 2007; Lindh *et al.*, 2008). We show that for biting midges some bacteria are trans-stadially transmitted throughout all life stages. However, the aquatic larval stages have a unique bacterial community compared to the terrestrial pupal and adult stages, which suggests that bacterial communities in life stages after metamorphosis are also influenced by terrestrial factors. This is in line with research on *Anopheles* mosquitoes which showed that the midgut microbial community was mostly dependent on environmental factors and individual life history. Environmental factors included seasonality, diet, larval breeding site, blood-feeding and genetic identity (Wang *et al.*, 2011; Gimonneau *et al.*, 2014; Tchioffo *et al.*, 2015; Akorli *et al.*, 2016; Novakova *et al.*, 2017; Mancini *et al.*, 2018).

For this study, we identified bacterial community composition in the abdomens of adult females, whereas for the other life stages the whole egg, larva or pupa was included in the analyses. It is known from mosquito research that bacterial communities can be different among organs such as salivary glands, reproductive organs, and the midgut (Jupatanakul *et al.*, 2014; Tchioffo *et al.*, 2015). Our conclusions do not include the specific location of bacterial communities in an organ, which hampers the assignment of possible functions of identified bacteria in the biting midge bodies. We sampled abdomens instead of midguts, as we expected that the gut bacterial community is the main contributor to the total bacterial communities in the abdomen of biting midges.

Bacterial communities in field-collected biting midges

Most of the gut bacterial communities were unique for the five biting midge species collected in wetland habitats in The Netherlands. Communities of gut bacteria were relatively stable among samples within each species, which suggests that species have their own unique gut bacterial communities. This concurs with our earlier results with the two lab-reared biting midges and shows that most species have their own adult female gut bacterial communities. However, individuals were collected with an adult trap and it was unknown from what larval habitat they originated. In addition, for all results obtained from field-collected biting midges it must be highlighted that their age was unknown. Our data of two lab-reared biting midges show a large effect of age (newly emerged adults versus six-day-old adults) on the gut bacterial community composition. Since all samples were obtained in the same manner for each species, and because pools of five individuals per sample were used, we do not expect that the unknown age or larval habitat origin will influence our conclusions.

In addition to the five wetland species, gut bacterial communities were determined of four Obsoletus group species (*C. chiopterus, C. dewulfi, C. obsoletus* s.s. and *C. scoticus*) originating from farm habitats in Sweden, The Netherlands and Italy. Both geographic location and species identity had an effect on bacterial communities found in the gut of those biting midges. These differences among species might be explained by their differences in habitat choice or host preference (Díaz-Sánchez *et al.*, 2018). The influence of geographical distance on the bacterial community was found for *C. scoticus*, but not for *C. obsoletus* sensu stricto. This indicates that the influence of environmental factors such as temperature and available food sources, on the bacterial community, is comparable to the influence of species identity. Core bacteria from *Pseudomonas, Bacillus,* Enterobacteriaceae and *Sphingomonas*, found in the gut of all farm-associated species from the three countries are expected to be important in the physiology of these vector species.

Similar to results in wild mosquitoes, we found that bacterial communities of most iting midge species are dominated by a small number of taxa (Osei-Poku *et al.*, 2012). However, results on differences in bacterial communities among biting midge species contrast to those in mosquitoes, as mosquitoes were found to have a relatively similar gut bacterial composition among species, but large differences among individuals within species (Osei-Poku *et al.*, 2012; Jupatanakul *et al.*, 2014). More in line with our results, another study showed that mosquito species identity is most defining for the bacterial community when they sampled over multiple years (Novakova *et al.*, 2017). Similarly, a cross-taxon analysis showed that bacterial composition was more similar within species than between species (Jones *et al.*, 2013). We used a pool of five individuals to analyse the bacterial community. This may explain why we find more similarity among samples and more differences among species than earlier work performed on individual mosquitoes.

Laboratory-reared and field-collected biting midges

To gain insight in the similarity of lab-reared and field-collected species, a comparison was made between the gut bacterial communities of 11 different species. The bacterial communities of the two lab-reared species were different from bacterial communities in nine field-collected species. This is in line with earlier studies that found significant differences in bacterial community composition for lab-reared and field-collected *C. sonorensis* biting midges and *Culex* mosquitoes (Campbell *et al.*, 2004; Duguma *et al.*, 2015). In the current study we did not compare the same species for both field and laboratory. However, the difference in bacterial communities among several species indicates that extrapolations of microbiota studies on lab-reared biting midges to field populations should be interpreted with caution, even when multiple species are used.

No common bacteria could be identified that were present in all 11 species. However, in at least 43% of the samples from each biting midge species, *Pseudomonas* bacteria were found, which suggests that it has a close association with biting midges in general. As mentioned earlier *Pseudomonas* are known to be common bacteria in wet environments and can be beneficial to insects in several ways (Parker *et al.*, 1977; Campbell *et al.*, 2004; Peck & Walton, 2006; Wang *et al.*, 2011; Alvarez *et al.*, 2012; Senderovich & Halpern, 2013; Chavshin *et al.*, 2015; Erram, 2016). It is therefore not surprising that, in our study, these bacteria were found in a wide range of biting midge species.

Distinct bacteria associated with biting midges

Earlier research identified *Corynebacterium*, *Propionibacterium*, *Brevibacterium, Staphylococcus* and *Micrococcus* bacteria as part of the bacterial communities found in biting midges (Parker *et al.*, 1977; Campbell *et al.*, 2004; Erram, 2016; Díaz-Sánchez *et al.*, 2018). It must be stressed that in the current study several of the ASVs belonging to these genera were identified as contaminants and excluded from the dataset before further analyses. Several of these genera are known to be commonly found as human skin bacteria and might actually be contaminations instead of core microbiota (Grice & Segre, 2011).

The gut of lab-reared *C. nubeculosus* adult females was dominated by *Asaia* bacteria. However, *Asaia* could also be found in several field-collected species. Bacteria from the genus *Asaia* were previously isolated from several *Anopheles* mosquito species from both larvae and adults (Favia *et al.*, 2007), and more recently *Asaia* was found in low abundance in field-collected adult *Culicoides* biting midges from the USA and Australia (Mee *et al.*, 2015; Erram, 2016). *Asaia* has been proposed as a suitable candidate for paratransgenic manipulation of mosquito vector competence against malaria (Favia *et al.*, 2008). In addition, *Asaia* was shown to have a strong mutual exclusion interaction with *Wolbachia* infection in several tissues of mosquitoes (Rossi *et al.*, 2015). It will therefore be interesting to further investigate the *Asaia* bacteria that are present in biting midges and their potential role for paratransgenic control of arboviruses.

Biting midge species identity largely reflected which bacterial taxon dominated their gut bacterial communities. In lab-reared species these bacteria were midgut-associated species, whereas endosymbiotic bacteria were found in high relative abundance in several field-collected species. Some of the dominant endosymbiotic bacteria in field-collected species such as *Cardinium, Rickettsia* and *Wolbachia* were already known to be associated with insects and can affect development time, longevity, reproduction and even vector competence (Campbell *et al.*, 2004; Chigira & Miura, 2005; Perlman *et al.*, 2006; Chiel *et al.*, 2007; Hedges *et al.*, 2008; Nakamura *et al.*, 2009; Iturbe-Ormaetxe *et al.*, 2011; Morag *et al.*, 2012; Lewis *et al.*, 2014; Mee *et al.*, 2015; Erram, 2016; Pagès *et al.*, 2017; Pilgrim *et al.*, 2017).

For *Cardinium,* three out of the nine field-collected biting midge species in The Netherlands were positive for these bacteria. It was the dominant bacterial species in the gut of *C. festivipennis* and *C. punctatus* which is in line with other studies that found *Cardinium* in several species of biting midges, including *C. festivipennis* and *C. punctatus*. A higher prevalence of *Cardinium* was found in livestock-associated biting midges than those collected from natural habitats (Nakamura *et al.*, 2009; Morag *et al.*, 2012; Lewis *et al.*, 2014; Mee *et al.*, 2015; Pagès *et al.*, 2017). The individuals collected for the current study originated from wetland (natural) habitats and had prevalence of *Cardinium* up to 99% of the total bacterial community. *Cardinium* is often located in the reproductive tissues and is known to be involved in reproduction alterations of arthropod hosts including parasitic wasps, mites and spiders, either through cytoplasmic incompatibility, parthenogenesis or feminization (Chigira & Miura, 2005; Chiel *et al.*, 2007). Currently *Cardinium* is classified into three groups (A-group, B-group and C-group) of which the C-group is specifically associated with *Culicoides* biting midges (Pagès *et al.*, 2017). Investigation into the role of *Cardinium* in reproductive alteration in biting midges will be an interesting next step.

Rickettsia were the most dominant bacteria in most samples of *C. alazanicus* with prevalence up to 91% of the total bacterial communities. In addition, *Rickettsia* were detected in five of the nine field species. Members of the genus *Rickettsia* are associated with human diseases such as typhus and Rocky Mountain spotted fever, but non-pathogenic *Rickettsia* have also been broadly associated with arthropods and invertebrates, including multiple *Culicoides* species (Campbell *et al.*, 2004; Erram, 2016; Pilgrim *et al.*, 2017). It is therefore suggested that *Rickettsia* are insect symbionts that are vertically transmitted in invertebrates and secondarily transmitted as pathogens to vertebrates. Similar to *Cardinium*, species of *Rickettsia* have been associated with reproductive manipulation through male-killing, parthenogenesis, and effects on fertility in insects (Perlman *et al.*, 2006), making this another interesting candidate for future studies.

The endosymbiotic bacterium *Wolbachia* was the dominant species found in *C. kibunensis* biting midges with up to 98% of the total gut bacterial communities in some samples. *Wolbachia* was less abundant, but nevertheless detected in five out of the nine field-collected species, which included the four species of the Obsoletus group. Our findings are in line with earlier studies that found *Wolbachia* in a range of *Culicoides* biting midge species, including *C. obsoletus* s.l., and *C. kibunensis*. *Wolbachia* was found with higher prevalence in biting midges from natural habitats compared to livestock premises, which is the opposite from prevalence of *Cardinium* (Lewis *et al.*, 2014; Mee *et al.*, 2015; Pagès *et al.*, 2017). *Wolbachia* bacteria are known to block the transmission of arboviruses in mosquitoes (Hedges *et al.*, 2008; Iturbe-Ormaetxe *et al.*, 2011). It is currently unknown to what extent *Wolbachia* affects virus infection in biting midges or *C. kibunensis* in particular.

In contrast to the relatively simple gut bacterial communities with only a few taxa in biting midges from wetland habitats, gut bacterial communities of adult *C. sonorensis* and Obsoletus group species (*C. chiopterus, C. dewulfi, C. obsoletus* s.s.*, C. scoticus*) showed more diversity. Interestingly, these species are recognized as more competent vectors of pathogens compared to *C. nubeculosus* and the wetland collected species. Whether a larger gut bacterial diversity is truly associated with higher vector competence remains an area for further research.

Both *Bacillus* and *Sphingomonas* were identified as important bacteria that commonly occurred in Obsoletus group species. *Bacillus* was found earlier in *C. imicola* and *C. sonorensis* field-collected samples (Parker *et al.*, 1977; Díaz-Sánchez *et al.*, 2018). It will be interesting to investigate if certain bacterial species such as *Bacillus* or *Sphingomonas* can be linked to increased infection rates or susceptibility of specific biting midge species. Either by introducing them into axenic lab-reared biting midges and determining changes in vector competence, or by identifying gut bacterial communities of field-collected individuals that were infected with a virus.

Introduction of specific (combinations of) bacteria in axenic and gnotobiotic biting midge species will provide important insights in how the gut of biting midges can be colonized and if this colonization is the result of initial introduction of a specific bacterial species, or with other factors that favour the growth of a specific bacterial species (Correa *et al.*, 2018; Guégan *et al.*, 2018; Valzania *et al.*, 2018). This knowledge can be used to further explore the functional role of biting midge microbiota and their influence on life history traits such as development rate, lifespan, fecundity and oviposition.

Conclusions

Our results show that metamorphosis is not only a key event in the development of the biting midge itself, but also for its midgut bacterial community composition, which changed significantly after pupation for both *C. nubeculosus* and *C. sonorensis*. Nevertheless, *Pseudomonas,* Burkholderiaceae and *Leucobacter* bacteria were transstadially transmitted and found throughout the biting midge life cycle. We show that lab-reared and field-collected biting midge species harbour unique gut bacterial communities, with only *Pseudomonas* as shared bacterium. Geographic distance and species identity determined the gut bacterial composition of field-collected biting midges. These differences in bacterial communities among species and countries might partly explain the observed inter- and intra-species variability in vector competence of biting midges. The presence of *Pseudomonas,* Enterobacteriaceae and *Sphingomonas* as core bacteria in Obsoletus group species suggests that they play a fundamental role in the biology of farm-associated European biting midges.

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Supplementary

Table S1. Gut bacterial diversity. Estimators of taxonomic diversity for gut microbiota of different life stages from two lab-reared species (*C. nubeculosus* and *C. sonorensis*), from five field-collected species (*C. alazanicus, C. festivipennis, C. kibunensis, C. pictipennis, C. punctatus*) from wetlands in The Netherlands and from Obsoletus group species (*C. chiopterus, C. dewulfi, C. obsoletus* s.s., *C. scoticus*) collected at farms in Sweden, The Netherlands and Italy. Average values (minimum – maximum) calculated for the number of samples per group are presented for Inverse Simpson, Shannon-Wiener Diversity and Shannon-Wiener Evenness.

Samples	No. of samples	Inverse Simpson Index	Shannon-Wiener Diversity	Shannon-Wiener Evenness
C. nubeculosus eggs	8	3.318 (1.004 – 6.977)	1.407 (0.019 – 2.572)	1.521 (1.015 – 2.035)
C. nubeculosus larvae	8	3.495 (1.003 – 6.295)	1.447 (0.014 – 2.212)	1.493 (1.011 – 1.956)
C. nubeculosus pupae	8	5.045 (1.151 – 13.014)	1.862 (0.407 – 3.120)	1.727 (1.306 – 2.403)
C. nubeculosus newly emerged	8	3.769 (1.010 – 7.717)	1.552 (0.044 – 2.808)	1.699 (1.034 – 2.221)
C. nubeculosus six-day-old	18	1.257 (1.011 – 2.070)	0.351 (0.046 – 0.837)	1.158 (1.036 – 1.332)
C. sonorensis eggs	7	2.851 (1.033 – 5.558)	1.301 (0.115 – 2.526)	1.573 (1.085 – 2.250)
C. sonorensis larvae	8	2.442 (1.007 – 8.640)	0.804 (0.031 – 2.722)	1.304 (1.024 – 1.812)
C. sonorensis pupae	8	5.339 (1.031 – 12.639)	1.827 (0.096 – 3.189)	1.674 (1.068 – 2.375)
C. sonorensis newly emerged	8	5.420 (2.085 – 7.953)	2.101 (0.904 – 2.626)	1.665 (1.184 – 1.962)
C. sonorensis six-day-old	10	2.437 (1.075 – 7.647)	1.070 (0.202 – 2.771)	1.522 (1.139 – 2.089)
C. alazanicus (NL wetland)	6	2.944 (1.203 – 9.739)	1.078 (0.374 – 2.848)	1.425 (1.208 – 1.771)
C. festivipennis (NL wetland)	6	1.953 (1.237– 2.311)	0.850 (0.447 – 0.987)	1.229 (1.130 – 1.475)
C. kibunensis (NL wetland)	7	1.399 (1.058 – 2.470)	0.650 (0.213 – 1.617)	1.451 (1.148 – 2.040)
C. pictipennis (NL wetland)	7	1.741 (1.005 – 4.127)	0.641 (0.020 – 2.044)	1.333 (1.016 – 1.887)
C. punctatus (NL wetland)	6	1.611 (1.017 – 3.323)	0.522 (0.065 – 1.451)	1.165 (1.049 – 1.285)
C. chiopterus (NL farm)	3	6.892 (1.604 – 11.043)	2.144 (1.033 – 2.880)	1.638 (1.549 – 1.752)
C. dewulfi (NL farm)	12	7.554 (1.217 – 18.273)	2.253 (0.554 – 3.184)	1.718 (1.266 – 2.091)
C. obsoletus s.s. (NL farm)	11	5.748 (1.022 – 12.301)	1.972 (0.087 – 3.237)	1.649 (1.067 – 2.629)
C. scoticus (NL farm)	11	3.983 (1.005 – 17.312)	1.216 (0.023 – 3.163)	1.475 (1.018 – 2.466)
Obsoletus group (NL farm)	37	5.902 (1.005 – 18.273)	1.852 (0.023 – 3.237)	1.619 (1.018 – 2.629)
C. dewulfi (SW farm)	1	2.413 (2.413 – 2.413)	1.545 (1.545 – 1.545)	1.942 (1.942 – 1.942)
C. obsoletus s.s. (SW farm)	5	3.814 (1.063 – 9.541)	1.534 (0.181 – 3.022)	1.764 (1.127 – 2.152)
C. scoticus (SW farm)	14	4.423 (1.009 – 17.198)	1.184 (0.038 – 3.199)	1.419 (1.030 – 2.083)
Obsoletus group (SW farm)	20	4.171 (1.009 – 17.198)	1.289 (0.038 – 3.199)	1.531 (1.030 – 2.152)
C. obsoletus s.s. (IT farm)	9	5.264 (1.014 – 13.956)	1.507 (0.058 – 2.936)	1.388 (1.045 – 1.756)
C. scoticus (IT farm)	9	7.988 (1.479 – 22.797)	2.382 (0.938 – 3.547)	1.758 (1.514 – 2.095)
Obsoletus group (IT farm)	18	6.626 (1.014 – 22.797)	1.945 (0.058 – 3.547)	1.573 (1.045 – 2.095)

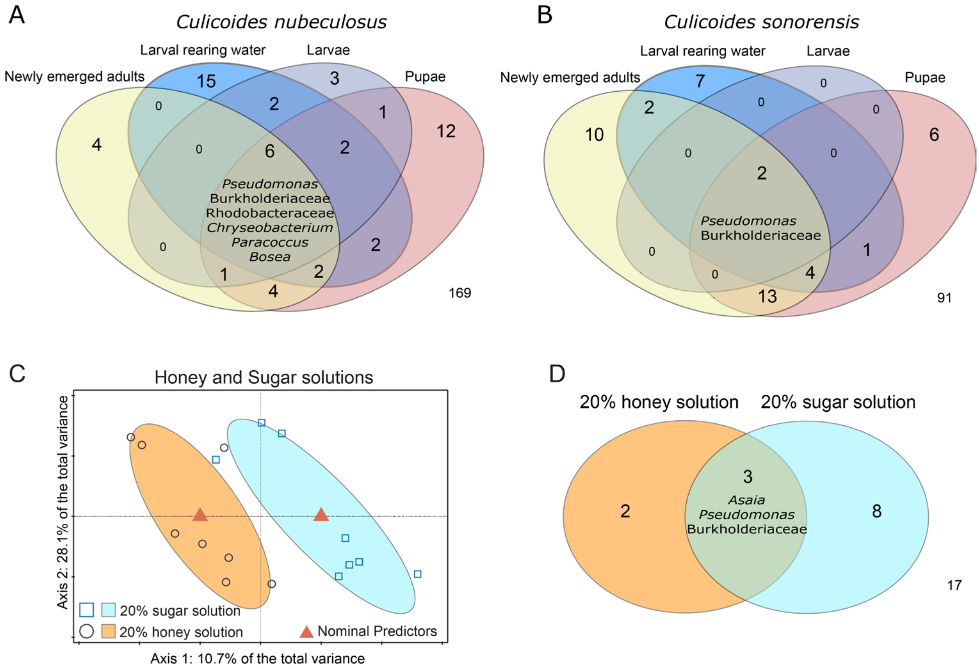
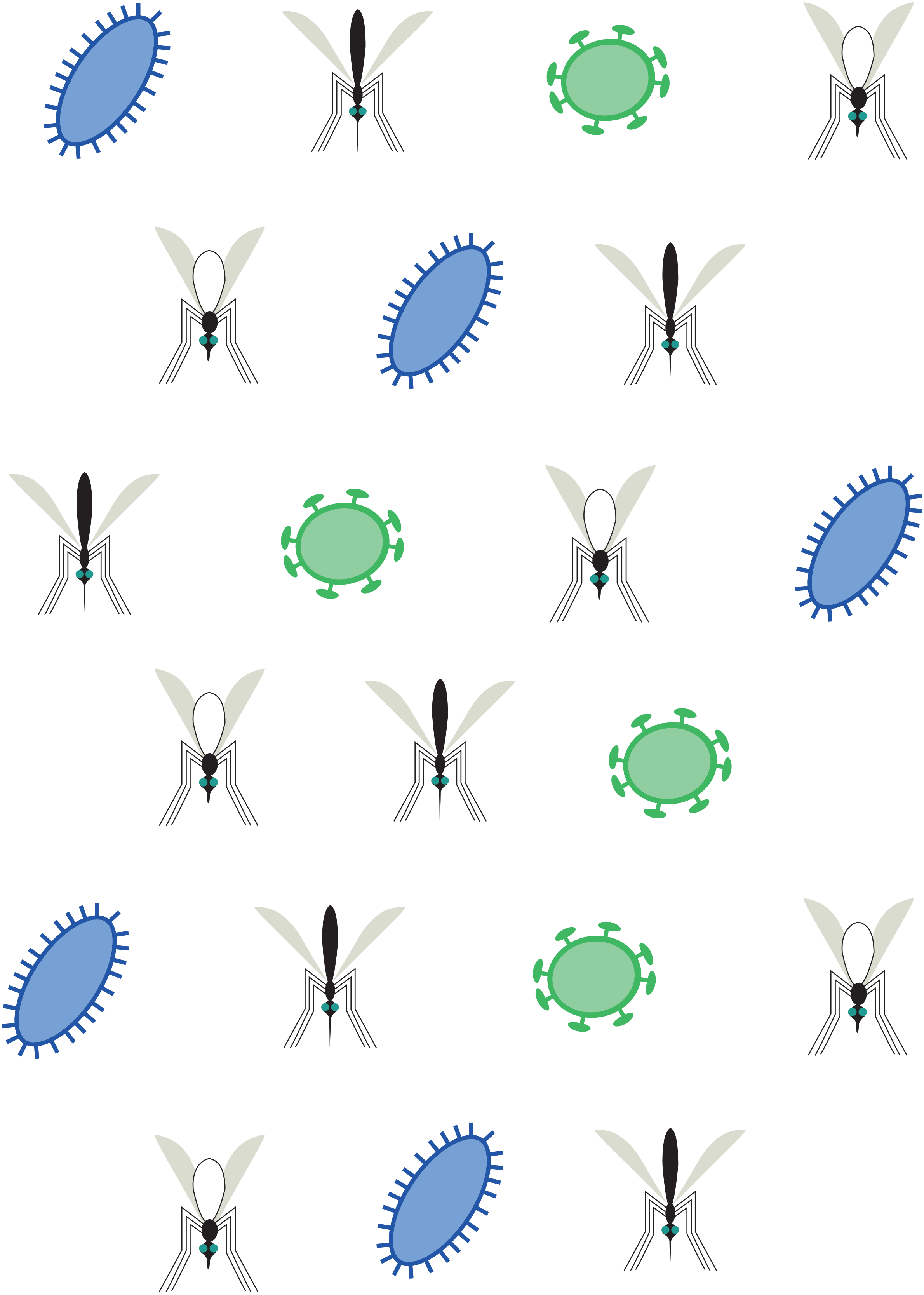


Figure S1. Venn diagrams and Redundancy analysis (RDA) illustrating overlap and differences of bacterial communities among life stages of *Culicoides* biting midges and environmental factors. Panel A: Venn diagram with number of bacterial ASVs specific and common among *C. nubeculosus* biting midge larvae (L4), pupae, newly emerged adults and their rearing habitat. Panel B: Venn diagram with number of bacterial ASVs specific and common among *C. sonorensis* biting midge larvae (L4), pupae, newly emerged adults and their habitat. Panel C: RDA of logarithm of the fraction of bacteria in six-day-old *C. nubeculosus* adults that were fed ad libitum on either 20% honey solution (orange) or 20% sugar solution (blue) (N = 16, DF = 1, F = 1.7, p=0.074). Ellipses show 66% confidence levels (±1 time the standard deviation). Panel D: Number of bacterial ASVs specific and common among six-day-old *C. nubeculosus* adults that were fed ad libitum on either 20% honey solution or 20% sugar solution. Names reflect the genera to which the ASVs belonged. The number outside the Venn-diagram indicates the number of ASVs that were excluded from the Venn-diagram based on the used thresholds. ASVs were only included in the Venn-diagram if they accounted for at least 0.1% of the total ASV-count in each sample and if they were present in at least 50% of the samples for each group.



Chapter 8

Impact of gut bacteria on the infection and transmission of pathogenic arboviruses by biting midges and mosquitoes

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Abstract

Tripartite interactions among insect vectors, midgut bacteria, and viruses, may determine the ability of insects to transmit pathogenic arboviruses. Here, we investigated the impact of gut bacteria on the susceptibility of *Culicoides nubeculosus* and *Culicoides sonorensis* biting midges for Schmallenberg virus, and of *Aedes aegypti* mosquitoes for Zika and chikungunya virus. Gut bacteria were manipulated by treating the adult insects with antibiotics. The gut bacterial communities were investigated using Illumina MiSeq sequencing of 16S rRNA, and susceptibility to arbovirus infection was tested by feeding insects with an infectious blood meal. Antibiotic treatment led to changes in gut bacteria for all insects. Interestingly, the gut bacterial composition of untreated *Ae. aegypti* and *C. nubeculosus* showed *Asaia* as dominant genus, which was drastically reduced after antibiotic-treatment. Furthermore, antibiotic-treatment resulted in relatively more *Delftia* bacteria in both biting midge species, but not in mosquitoes. Antibiotic-treatment and subsequent changes in gut bacterial communities significantly increased infection rates of *C. nubeculosus* with Schmallenberg virus. For *C. sonorensis* a similar increasing trend of infection rate was observed, whereas we did not find any changes in infection rates for *Ae. aegypti* mosquitoes with Zika or chikungunya virus. We conclude that the effect of gut bacteria on arbovirus infection is specific for each vector, virus, and bacterial species combination.

Keywords: Vector competence, metagenomics, microbiome, *Aedes aegypti*, *Culicoides nubeculosus*, *Culicoides sonorensis*, Schmallenberg virus, Zika virus, chikungunya virus

Introduction

Symbiotic micro-organisms play a key role in the physiology of their insect hosts (Crotti *et al.*, 2009; Jupatanakul *et al.*, 2014). For example, micro-organisms that reside in the insect gut provide extra nutrients to insects with a poor diet, such as aphids and termites (Gündüz & Douglas, 2009; Brune, 2014). Furthermore, gut bacteria are important in insect development and fitness. Developmental time was delayed and egg production was reduced in mosquitoes reared free of living bacteria (Correa *et al.*, 2018; Valzania *et al.*, 2018). Of particular interest is the tripartite interaction among insect vector, midgut bacteria, and the pathogens that those vectors may transmit (Cirimotich, Ramirez*, et al.*, 2011; Hegde *et al.*, 2015). Midgut microbiota can provide direct protection against pathogens that enter the insect body as was shown for Triatomine bugs and malaria mosquitoes (Azambuja *et al.*, 2004; Patricia Azambuja *et al.*, 2005). These beneficial bacteria have already been used in the control of arthropod-borne pathogens. Genetically modified bacterial symbionts such as *Asaia*, *Pantoaea agglomerans*, *Rhodococcus rhodnii* and *Serratia* have been used to combat pathogen transmission by vectors (Durvasula *et al.*, 1997; Favia *et al.*, 2007; Bisi & Lampe, 2011; Apte-Deshpande *et al.*, 2012; Stathopoulos *et al.*, 2014; Wang *et al.*, 2017; Guégan *et al.*, 2018). In addition, the endosymbiotic bacterium *Wolbachia* is a well-studied example of how a microbe can disrupt the transmission of arboviruses by mosquitoes (Moreira *et al.*, 2009; Iturbe-Ormaetxe *et al.*, 2011; Audsley *et al.*, 2017; Amuzu *et al.*, 2018). However, a study conducted on the interaction of *Wolbachia* and West Nile Virus in *Culex* mosquitoes showed increased virus titers in the presence of *Wolbachia* (Dodson *et al.*, 2014). This indicates that the impact of bacteria on virus transmission is context dependent.

Although the effects of *Wolbachia* and several genetically modified bacteria on arbovirus transmission have been extensively studied (Moreira *et al.*, 2009; Iturbe-Ormaetxe *et al.*, 2011; Bourtzis *et al.*, 2014), thus far only few studies have investigated the role of symbiotic midgut bacteria on pathogen transmission (Guégan *et al.*, 2018). Pathogens are ingested together with a blood meal and have to overcome the midgut barrier before they can infect the insect body. It is hypothesized that midgut bacteria have an effect on pathogen infection either mechanically or via activation of the vector's immune system. While the interaction of mosquito midgut bacteria with malaria parasites has been studied in more detail, relatively few studies have investigated the role of microbiota in transmission of arboviruses. Previous reports have shown an increased replication of arboviruses after elimination of the midgut bacteria (Xi *et al.*, 2008; Apte-Deshpande *et al.*, 2012; Ramirez *et al.*, 2012; Apte-Deshpande *et al.*, 2014; Ramirez *et al.*, 2014; Barletta *et al.*, 2017). Elimination of the mosquito midgut bacteria seems to reduce basal levels of antiviral immune response pathways such as the Toll pathway, leading to increased susceptibility to arbovirus infection. Although these studies show increased viral titers and reduced immune response pathways, they did not report on infection rate or transmission efficiency, which are important (quantitative) components of vector competence. Knowledge on the impact of the midgut bacterial community on the proportion of vectors that can transmit an arbovirus (vector competence) is currently lacking.

The aim of this study was to investigate the effect of gut bacteria on infection and transmission of arboviruses by their vector. As a model system, we selected three arboviruses belonging to different families, namely Schmallenberg virus (SBV; family Peribunyaviridae, genus *Orthobunyavirus*), Zika virus (ZIKV; family Flaviviridae, genus *Flavivirus*) and chikungunya virus (CHIKV; family Togaviridae, genus *Alphavirus*). These arboviruses are transmitted by biting midges (SBV by *Culicoides nubeculosus* and *Culicoides sonorensis*), or mosquitoes (ZIKV and CHIKV by *Aedes aegypti*). We sequenced the bacterial content in the gut system of both untreated and antibiotic-treated adult females of the three vector species, to identify changes in the microbial community. Subsequently, we determined infection rates and transmission efficiencies of untreated and antibiotic-treated virus-exposed females. In addition, virus titers were compared between the two treatments to investigate the effect of the gut microbial communities on the replicative fitness of the viruses.

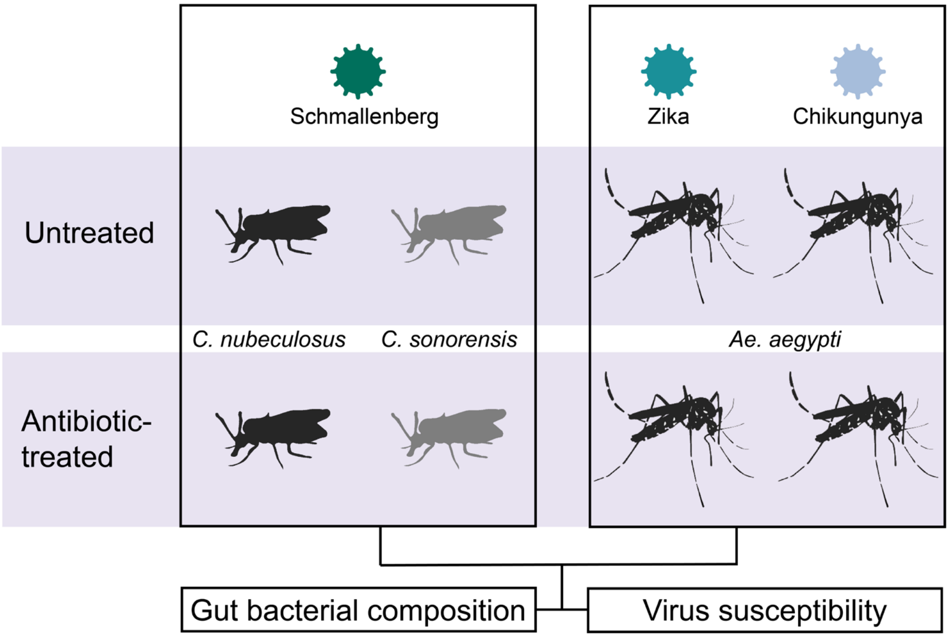

Figure 1. Overview of experimental design. Schmallenberg virus was used for infection of *Culicoides nubeculosus* and *C. sonorensis* biting midges, whereas Zika and chikungunya virus were used for infection of *Aedes aegypti* mosquitoes. All three vector species were divided in an untreated and an antibiotic-treated group. The gut bacterial communities of the three vector species for the untreated and antibiotic-treated groups were identified via 16S rRNA sequencing.

Materials & Methods

To investigate the role of insect midgut bacterial communities on arbovirus infection and transmission, we selected three vector species and three viruses (Fig. 1). The microbial gut communities of antibiotic-treated and untreated adult females was identified and their susceptibility to the respective viruses was tested. Two biting midge species (*C. nubeculosus* and *C. sonorensis*) were both exposed to SBV. One mosquito species (*Ae. aegypti*) was exposed to ZIKV or CHIKV. Adult female insects of each species were divided into untreated (control) and antibiotic-treated groups.

Insect vectors

Culicoides nubeculosus were provided by The Pirbright Institute, Pirbright laboratories, United Kingdom (Boorman, 1974), and were maintained at 23±1 °C with 16:8 light:dark cycle and 60% relative humidity. *Culicoides sonorensis* were provided by the Arthropod-Borne Animal Diseases Research Unit, USDA-ARS and were maintained at 25 °C with 16:8 light:dark cycle and 70% relative humidity. Similar rearing protocols were used for both biting midge species (chapter 9). Briefly, eggs were transferred to trays with filter wool pasted to the bottom (Europet Bernina International, Gemert-Bakel, The Netherlands), filled with tap water and two drops of Liquifry No.1 (Interpet, Dorking, United Kingdom). Larvae were fed with a 1:1:1 mixture of bovine liver powder (MP biomedicals, Irvine, CA, US), ground rabbit food (Pets Place, Ede, The Netherlands), and ground koi food (Tetra, Melle, Germany). *Culicoides nubeculosus* larvae were additionally fed with nutrient broth No. 1 (Oxoid, Hampshire, UK). Pupae were transferred to moist emergence cups that were placed in plastic buckets (diameter: 12.2 cm, height: 12.2 cm; Jokey, Wipperfürth, Germany) and closed with netting on the top through which the biting midges could feed. Emerged adults were provided with 6% glucose solution *ad libitum*. Bovine blood (Carus, Wageningen, The Netherlands) was provided through a Parafilm M membrane using the Hemotek PS5 feeding system (Discovery Workshops, Lancashire, United Kingdom) for egg production.

Aedes aegypti from the Rockefeller strain (Bayer AG, Monheim, Germany) were used in all mosquito experiments. The mosquito colony was maintained as described previously (Göertz *et al.*, 2017). In short, mosquitoes were kept at 27±1 °C with 12:12 light:dark cycle and 70% relative humidity. Adult mosquitoes were maintained on 6% glucose solution *ad libitum*. Human blood (Sanquin Blood Supply Foundation, Nijmegen, The Netherlands) was provided through a Parafilm M membrane using the Hemotek PS5 feeding system for egg production. Drought-conditioned eggs were transferred to transparent square larval holding trays (19 x 19 x 20 cm; Jokey), filled for approximately one-third with tap water and three drops of Liquifry No. 1. Hatched larvae were fed with Tetramin Baby fish food (Tetra). Larval trays were closed with fine-meshed netting to allow adult mosquitoes to emerge inside larval trays. Twice a week, adults were aspirated from larval trays and collected in Bugdorm-1 insect rearing cages (30 cm x 30 cm x 30 cm; Bugdorm, Taiwan, China).

Antibiotic treatment

Approximately 100-200 *C. nubeculosus* and *C. sonorensis* pupae were collected during three consecutive days and placed in a Petri dish containing moistened cotton wool and filter paper in separate buckets (diameter: 12.2 cm, height: 12.2 cm; Jokey). For a period of 6 days they were allowed to hatch and had direct access to 6% glucose solution (untreated group), or 6% glucose solution containing a combination of 10 µg/ml penicillin and 10 µg/ml streptomycin (PenStrep) (Duchefa biochemie B.V., Haarlem, The Netherlands) (antibiotic-treated group) (Touré *et al.*, 2000). Penicillin was chosen because it is a broad-spectrum antibiotic against gram-positive bacteria, and streptomycin was chosen because it is a broad-spectrum antibiotic against gram-negative bacteria. Biting midges in the antibiotic-treated group were allowed to feed on a glucose solution with antibiotics for three to six days before being transferred to the Biological Safety Level 3 (BSL3) facility at Wageningen University & Research, where arbovirus infections were performed. Antibiotic-treatment was continued throughout the duration of the experiments.

Aedes aegypti adults were collected from larval trays and divided into two groups of approximately 100-200 mosquitoes in Bugdorm-1 cages. One cage was maintained on 6% glucose solution (untreated group), whereas the other cage was maintained on 6% glucose solution with 20 U/ml penicillin (Sigma-Aldrich, Saint Louis, MO, United States) and 20 µg/ml streptomycin (PenStrep) (antibiotic-treated group; Sigma-Aldrich) for four days (Ramirez *et al.*, 2012). Females were then transferred to plastic buckets (diameter: 12.2 cm, height: 12.2 cm; Jokey) and transported to the BSL3 facility for arbovirus infection studies.

Taxonomical identification of gut bacterial populations

Sample preparation

To gain insight in the effect of the antibiotic treatment on gut bacterial community composition, biting midges and mosquitoes were dissected and their gut bacterial communities were identified. Prior to dissection, biting midges were anesthetized by freezing for 15 to 30 minutes at -20°C. To remove external bacterial contamination, each biting midge was dipped in 70% ethanol for 10 sec, in 5% sodium hypochlorite solution for 60 sec, and finally rinsed in 70% ethanol for 30 sec (chapter 7; Gusmão *et al.*, 2007; Osei-Poku *et al.*, 2012; Nayduch *et al.*, 2015). After cleaning, pools were made under aseptic conditions from dissected abdomens from five untreated or five antibiotic-treated females in a 2 ml screw cap micro tube (Sarstedt) with a 4 mm borosilicate glass bead (Sigma-Aldrich). In total, 18 replicate pools were prepared for untreated and antibiotic-treated *C. nubeculosus*, and 10 replicate pools for untreated and antibiotic-treated *C. sonorensis*, resulting in a total of 56 pools.

Similar to the biting midges, mosquito midgut bacteria were investigated. Mosquitoes were treated in a similar manner as described above. Midguts were dissected and pooled from five untreated and five antibiotic-treated females, under aseptic conditions. Selected mosquitoes were anesthetized on ice, dipped in 70% ethanol for 10 seconds, and then rinsed in phosphate buffered saline (PBS) for 10 seconds. Midguts were dissected in a droplet of PBS using forceps, under the dissecting microscope. Five midguts per treatment were pooled in a 2 ml screw cap micro tube with a 4 mm borosilicate glass bead. In total, 12 replicate pools were prepared for untreated and antibiotic-treated *Ae. aegypti* females, resulting in a total number of 24 pools.

DNA extraction protocol

Midgut pools were placed in Precellys Evolution tissue homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) and homogenized twice at 7800 rpm for 15 sec. The VWR Mag-Bind Tissue DNA KF 96 Kit (Omega bio-tek, Norcross, GA, United States), was used for DNA extraction of bacterial populations as per the manufacturer's protocol. After extraction, 100 µl was transferred from the elution plate into small Eppendorf tubes and stored at -20 °C until further processing.

qPCR

Midgut bacterial loads were quantified for each sample by SYBR Green real-time PCR (Thermo Fisher Scientific, Waltham, United States) (chapter 7; Fierer *et al.*, 2005). Five µl of each sample was added to a master mix of 20 µl consisting of 0.12 µl 100 µM Eub338f forward primer, 0.12 µl 100 µM Eub518r µl reverse primer, 10 µl Takara 2x, 0.4 µl ROX2, and 4.36 µl Milli-Q water. The qPCR program was run at 50 °C for 2 min, 95 °C for 10 min, then 40 cycles of 95 °C for 15 sec and 50 °C for 1 min, followed by 95 °C for 15 sec, 50 °C for 1 min, 95 °C for 30 sec and finally 50 °C for 15 sec.

Based on the qPCR results, subsamples of the dataset were selected for 16S rRNA PCR at different number of cycles. These samples were run on gel and the intensity of electrophoresis was used to visually estimate if bacterial DNA load after PCR was comparable among samples. This process was repeated with adjusted numbers of PCR cycles until comparable DNA load was achieved. Samples were then subjected in triplicate to PCR with 5 µl sample and 20 µl master mixture consisting of 1.2 µl dNTP (5mM), 6 µl 5xQ5 reaction buffer, 0.15 µl 16S V4 515F forward primer (100 µM), 0.15 µl 16S V4 806R reverse primer (100 µM), 0.3 µl Q5 HF DNA polymerase, and 14.7 µl Milli-Q water (Caporaso *et al.*, 2011)2011. Samples were run on Verity PCR machines (Thermo Fisher Scientific, Waltham, United States) with the following program; 98 °C for 30 sec, 98 °C for 10 sec, 50 °C for 30 sec, 72 °C for 30 sec, 72 °C for 2 min and 4 °C until the program was stopped. The number of cycles varied per sample but all were between 16 and 29 cycles. Obtained amplicons of the three PCR replicates per sample were pooled and stored at -20 °C before further processing.

Sequencing and preparation of data

Samples were sequenced on an Illumina MiSeq platform (Next Generation Sequencing facilities, Wageningen University & Research, Wageningen, The Netherlands). Resulting reads were analysed with QIIME2 (version 2018.8; https://qiime2.org; chapter 7; Caporaso *et al.*, 2010; Bolyen *et al.,* 2018). All forward and reverse reads were demultiplexed and linked to sample-IDs. Sequence run specific quality control, merging of forward and reverse reads, removal of 16S V4 primer sequences and of chimeric sequences was performed with the DADA2 package as QIIME2 plugin (Callahan *et al.*, 2016). DADA2 grouped unique sequences equivalent to operational taxonomic unit (OTU) clustering at 100% similarity, resulting in an abundance table (feature table) of the amplicon sequence variants (ASVs) and a file with the unique sequences (rep-seqs). Advantages of the new ASV approach compared to OTU clustering at 97% similarity have been discussed previously (Callahan *et al.,* 2017). At first, sequences were aligned with MAFFT plugin (Katoh & Standley, 2013) and highly variable positions in alignment were masked (Lane, 1991) to reduce noise in the phylogenetic tree. FastTree plugin (Price *et al.*, 2010) was used to create an unrooted tree of the unique sequences. The tree was rooted at midpoint of the longest tip-to-tip distance.

Taxonomy was assigned with confidence threshold 0.8 to the unique sequences with Naive Bayes classifier pre-trained on the Silva database release "132 16S V4 region", with QIIME2 classifier plugin (https://docs.qiime2.org/; (Pedregosa *et al.,* 2011; Quast *et al.*, 2012; Bokulich *et al.*, 2018). The ASV abundance table was additionally filtered before further analyses. All sequences were removed that were not classified (unassigned at Kingdom taxa level), or classified as Eukaryotes, plant mitochondria or chloroplasts, as well as all ASVs without any phylum classification. Very low abundant ASVs with a total count below 10 were also removed as an additional noise reduction before further analyses. For analyses performed in R, the QIIME2 data was extracted into abundance or feature tables and converted from BIOM HDF5 to JSON format (McDonald *et al.,* 2012).

Negative control samples

Negative control samples (N = 14) were included that followed the complete protocol from DNA extraction to sequencing. These samples contained no insect material but did generate bacterial sequences. Such contaminants can originate from reagents used in the DNA extraction, PCR or next-generation sequencing library preparation, as well as from human skin, oral or respiratory microbiota (Knights *et al.*, 2011; Lazarevic *et al.*, 2016). The 14 samples contained 907 ASVs with a count of 204,153. After filtering of low abundant ASVs a total of 81 ASVs with a count of 176,225 remained. To identify true contaminants an occurrence threshold of 20% was used which means that an ASV was present in at least 3 out of the 14 negative control samples. In addition, the selected contaminants together had to contribute 99% to the total fraction counts. A total of 51 ASVs with a count of 140,573 were recognized as true contaminations and filtered from the complete dataset before further analyses. Identified contaminants consisted of several common skin bacteria such as *Corynebacteria*, *Propionibacteria, Staphylococci* and *Micrococcus* (Grice & Segre, 2011). Together these skin associated ASVs comprised 20% (28,562 / 140,573) of the total count in the negative controls.

Viruses

SBV was obtained from Wageningen Bioveterinary Research (Lelystad, The Netherlands) as passage three (P3) bovine isolate (B-SBV). Two additional passages, P4 and P5, were grown on *Aedes albopictus* C6/36 cells (ATCC, Manassas, United States, CRL-1660) in Leibovitz-15 (L-15) growth medium (Gibco, Carsbad, CA, United States) supplemented with 10% fetal bovine serum (FBS), 2% tryptose phosphate broth (Gibco), and 1% nonessential amino acids (Gibco), at 27 °C. Virus-containing supernatants were harvested at 5 days post inoculation and stored in aliquots at -80 °C. The P4 stock titer was determined by endpoint dilution assays (EPDA) on African green monkey kidney Vero E6 cells (ATCC CRL-1586). Virus titers were determined using the Reed & Muench algorithm (Reed & Muench, 1938).

ZIKV Suriname strain P4 stock, as described previously by Göertz *et al.* (2017), was used to grow a P5 stock on Vero cells and was used in all mosquito infection experiments. Vero cells were cultured in Dulbecco's modified Eagle medium (HEPES-DMEM; Gibco) supplemented with 10% FBS, at 37 °C and 5% CO_2. A T75 flask (Greiner Bio-One, Kremsmünster, Austria) pre-seeded with Vero cells was inoculated with ZIKV P4, and incubated for 3 days. Supernatant was harvested and stored in aliquots at -80 °C. The P5 stock titer was determined by EPDA, as above, on Vero cells.

Chikungunya strain 37997 was produced as previously described (Göertz *et al.*, 2017). A T75 flask pre-seeded with C6/36 cells was inoculated with CHIKV P1, and incubated for 3 days at 28 °C. Supernatant was harvested and stored in aliquots at -80 °C. The P2 stock titer was determined by EPDA, as above, on Vero cells.

Virus infections

Untreated and antibiotic-treated female biting midges were allowed to feed on an infectious blood meal containing SBV, whereas female mosquitoes were allowed to feed on an infectious blood meal containing either ZIKV or CHIKV. For each virus, a 1:1 dilution was prepared by adding an equal amount of bovine blood to SBV stock (average titer in blood-meal: 2.5×10^6), or human blood to either ZIKV stock (titer in blood-meal: 4.0×10^4) or CHIKV stock (titer in blood-meal: 2.5×10^8). These virus titers were deliberately selected based on pilot experiments to obtain intermediate infection rates to facilitate observations of both negative and/or positive effects of the midgut bacteria on virus infection rates. Bovine blood was verified for absence of SBV neutralizing-antibodies before the experiment started. The infectious blood-meal was provided through a Parafilm M membrane using the Hemotek PS5 feeding system, at 24±1 °C and 70% relative humidity. Biting midges were fed in the dark, whereas mosquitoes were fed under light conditions. After 1 hour, biting midges and mosquitoes were anesthetized with 100% CO_2, placed on a CO_2-pad (Genesee Scientific, San Diego, United States), and fully engorged females were selected and placed back in the holding bucket. Biting midges were maintained at 25 °C for 10 days and provided with 6% glucose solution *ad libitum* (untreated). Biting midges in the antibiotic-treated group were continuously fed on the glucose solution with PenStrep (antibiotic-treated). Engorged female mosquitoes were maintained at 28 °C for 10 days, and were provided with 6% glucose solution *ad libitum*.

Infection and transmission

Ten days post feeding, biting midges were anesthetized with 100% CO_2 and maintained on a CO_2-pad. Females were individually transferred to a 1.5 ml Safe-Seal micro tube (Sarstedt, Nümbrecht, Germany) containing 0.5 mm zirconium beads (Next Advance, Averill Park, NY, United States), and stored at -80 °C until further processing. The whole procedure was replicated three times, which resulted in a total number of 196 untreated (N_1 = 79, N_2 = 49, N_3 = 68) and 275 antibiotic-treated (N_1 = 114, N_2 = 92, N_3 = 69) C. *nubeculosus*, and 44 untreated (N_1 = 20, N_2 = 10, N_3 = 14) and 47 antibiotic-treated (N_1 = 19, N_2 = 5, N_3 = 23) *C. sonorensis*.

Ten days post feeding, mosquitoes were anesthetized with 100% CO_2 and maintained on a CO_2-pad to remove their legs and wings with forceps. Mosquito saliva was then collected by inserting the proboscis into a 200 µl yellow pipet tip (Greiner Bio-One) containing 5 µl of a 1:1 solution of 50% glucose solution and FBS. After at least 45 minutes, the mosquito body (head, thorax and abdomen) was transferred to a 1.5 ml Safe-Seal micro tube containing 0.5 mm zirconium beads. The saliva sample was transferred to a 1.5 ml micro tube (Sarstedt) containing 55 µl 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered DMEM (HEPES-DMEM) supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), fungizone (50 µg/ml; Invitrogen, Carlsbad, United States) and gentamycin (50 µg/ml; Gibco). All samples were stored at -80 °C until further processing. This whole procedure was replicated four times for both ZIKV and CHIKV, with N = 25 mosquito body and saliva samples per replicate for each of the four treatments.

Frozen biting midge and mosquito bodies were homogenized for two minutes at maximum speed in a Bullet Blender Storm (Next advance, Averill Park, NY, United States), centrifuged briefly, and re-suspended in 100 µl of fully supplemented HEPES-DMEM. Samples were blended again for two minutes at maximum speed, and centrifuged for two minutes at 14,500 rpm in an Eppendorf minispin plus (Eppendorf, Hamburg, Germany). Mosquito saliva samples were thawed at room temperature. In total, 30 µl of each body or saliva sample was used to inoculate a monolayer of pre-seeded Vero cells in a 96 wells plate. On each plate, diluted virus stock or infectious blood mixture was included as positive controls and wells to which no sample was added were included as negative controls. After 2-3 hours, the inoculum was removed and replaced by 100 µl of fully supplemented HEPES-DMEM. Wells were scored for virus induced cytopathic effect (CPE) at three and six days post inoculation, with full CPE being observed at the latter time point. Virus titers of infected biting midge bodies and of mosquito body and saliva samples were determined by EPDA on Vero E6 cells (Göertz *et al.*, 2017). If less than three wells in the first row showed CPE, the titer could not be calculated because the sample contained less than 1000 $TCID_{50}$ per ml.

Statistical analysis

The difference in bacterial communities between untreated and antibiotic-treated insects (biting midges or mosquitoes) was tested using a permutation test (999 permutations) based on a redundancy analysis (RDA) of taxa on the treatment factor using Canoco 5.11 (ter Braak & Šmilauer, 2018). All seven taxonomic levels were used simultaneously in these analyses, obtained by summing the ASV counts to the taxon levels kingdom (Bacteria and Archaea), phylum, class, order, family, genus, and species. In the analysis, the resulting counts were divided by the library size and the resulting fractions were log-transformed after addition of 0.001, to avoid problems with zero counts. The value 0.001 was chosen as its inverse is close to the smallest library size and gives a reasonably symmetric distribution of residuals. The approach has the advantage of yielding one test of significance instead of several level-specific tests. Selection of differentially expressed taxa was based on the percentage fit due to the treatment factor (in our case antibiotic treatment).

In addition, we conducted a univariate test using the log transformed fractions per taxon, to identify taxa that were correlated with untreated or antibiotic-treated samples. Univariate p-values were calculated with both Welch's two-sample t-test (two-sided) and its permuted version. The null distribution of the permuted t-test was calculated with 9,999 permutations with the function perm.t.test from the R package deducer. Given the correspondence between the p-values of these two methods, the false discovery rate (FDR, Benjamini-Hochberg correction) was based on the p-values of the (parametric) Welch's t-test. The FDR was calculated across all taxa levels together and per taxon level. Alpha diversity indices were calculated for Shannon-Wiener Diversity (H'), the Inverse Simpson Idex (D2 or N2), and the Shannon-Wiener Eveness index based N1/N2, where N1= exp(H') and N2 = Inverse Simpson Index using the VEGAN version 2.9.2. package (Oksanen *et al.*, 2009) in the statistical software package R version 3.5.0 (R Development Core Team, 2015, 2017).

Chi-square tests were used to test for the effect of antibiotic treatment on infection rate and transmission efficiency. For biting midges, only infection rates were determined, whereas both infection rates and transmission efficiency were determined for mosquitoes. Infection rate and transmission efficiency were calculated, respectively, by dividing the number of female vectors with virus-infected whole body (infection) or virus-infected saliva (transmission) by the total number of alive female vectors tested in the respective treatment, and multiplied by 100. Mann-Whitney U tests were used to test for the effect of antibiotic treatment on virus titers of body or saliva samples. All statistical analyses were done with the statistical software package R (R Development Core Team, 2015, 2017).

Results

Gut bacterial communities

To gain insight in the effect of the antibiotic treatment on the composition of gut bacterial communities, the identities of gut bacteria populations in adult female *C. nubeculosus*, *C. sonorensis* and *Ae. aegypti* were determined by high throughput 16S rRNA gene sequencing before blood-feeding. In addition, to uncover the role that specific gut bacteria may play in virus infection, bacterial species that were significantly different between untreated and antibiotic-treated females were determined by Redundancy analyses (RDA).

The communities of gut bacteria significantly differed between untreated and antibiotic-treated groups for all three vector species ($p<0.01$; Fig. 2A, C, E) The first principal component (PC), reflecting the difference between the bacterial communities of untreated and antibiotic-treated mosquitoes or biting midges, could explain a large part of the total variance (Fig. 2A, C, E). There was a significant difference between gut bacterial communities of untreated and antibiotic-treated for *C. nubeculosus* ($p=0.001$, Fig. 2A, the first PC explained 49% of the total variation), *C. sonorensis* ($p=0.001$, Fig. 2C, the first PC explained 14% of the total variation), and *Ae. aegypti* ($p=0.001$, Fig. 2E, the first PC explained 22% of the total variation).

After antibiotic treatment, a clear shift in gut microbial community was observed in *C. nubeculosus* and *Ae. aegypti* (Fig. 2B, F). Untreated samples of these two species were dominated by a single ASV that had a relative frequency of 34% to 98% of the total bacterial community (Fig. 2B, F). This ASV was identified as gram-negative *Asaia* bacterium (Phylum: Proteobacteria; family: Acetobacteraceae). The gut bacterial community of *C. nubeculosus* and *Ae. aegypti* that were treated with antibiotics still contained *Asaia*, but only up to 3% of the total bacterial community. A shift in midgut bacterial species was less evident for *C. sonorensis*, which overall showed more variation in bacterial communities in both the untreated and antibiotic-treated groups (Fig. 2D). Interestingly, the diversity of bacteria was higher for all three insect species after antibiotic-treatment compared to the untreated group (Table S2).

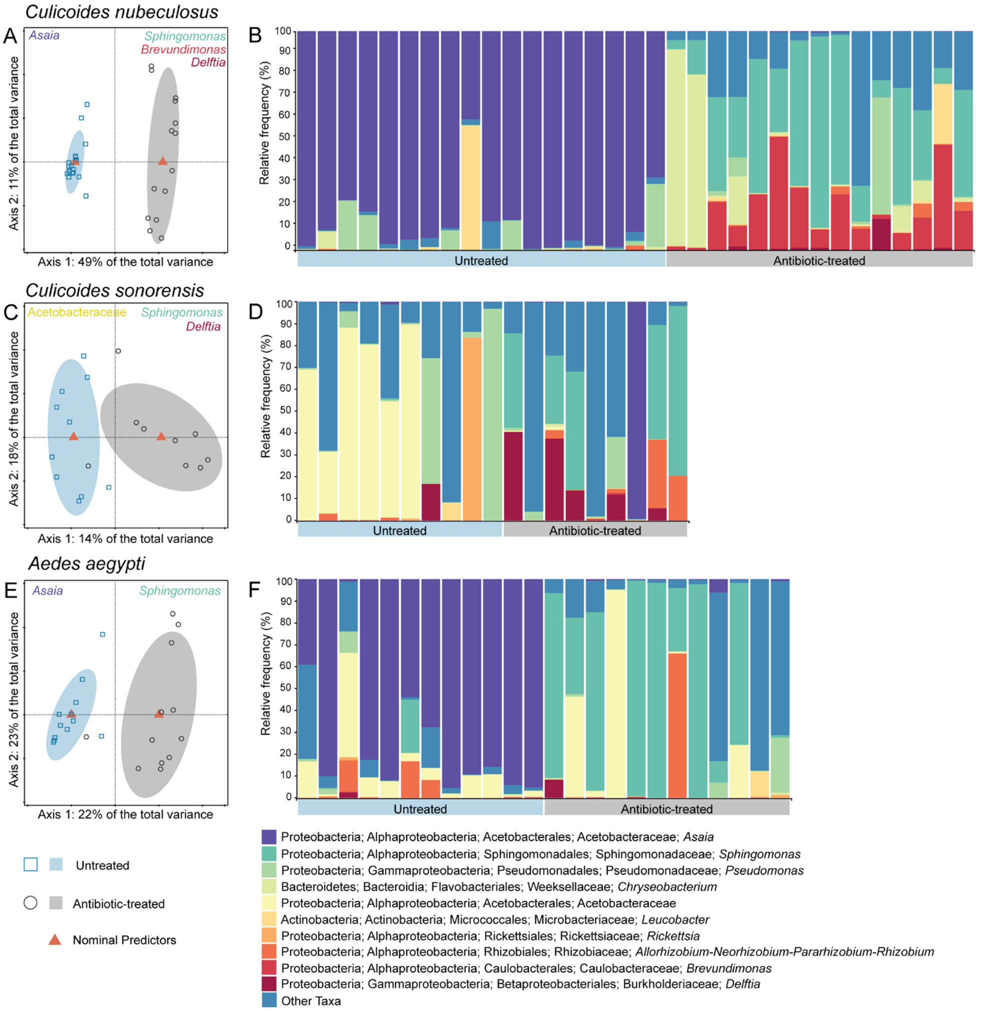

Figure 2. Redundancy analysis (RDA) plots and most abundant taxa of bacterial communities in untreated and antibiotic-treated biting midges and mosquitoes. RDA of logarithm of the fraction of bacteria in untreated and antibiotic-treated females of *Culicoides nubeculosus* (Panel A; N = 33, DF = 1, F = 30.0, *p*=0.001), *C. sonorensis* (Panel C; N = 19, DF = 1, F = 2.7, *p*=0.001) and *Aedes aegypti* (Panel E; N = 24, DF = 1, F = 6.1, *p*=0.001). Ellipses show 66% confidence levels (±1 time the standard deviation). A maximum of three taxa correlated with the untreated or antibiotic-treated groups are named at the top of panels A, C and E for each species. Panels B, D and F. Taxa-plots at genus-level, on the relative frequency for each taxon, of the total number of midgut bacteria in the community composition are presented. The 10 most abundant bacterial taxa are presented for midgut bacterial communities in *C. nubeculosus, C. sonorensis* and *Ae. aegypti*. Less abundant taxa were grouped as 'Other taxa' to increase visualization for the taxa plots. Each bar represents the relative frequency of bacterial taxa in one pool of five abdomens.

The family Acetobacteraceae was associated with each untreated group of insects, and more specifically for both *C. nubeculosus* and *Ae. aegypti* the genus *Asaia* within the Acetobacteraceae family. The antibiotic-treated groups for all three vector species were represented by the presence of bacteria in the *Sphingomonas* genus when compared to untreated groups. In addition, *Delftia* bacteria were correlated with antibiotic-treated compared biting midges (Fig. 2).

Infection rates and transmission efficiency

Vector competence was determined for untreated and antibiotic-treated biting midges and mosquitoes to gain insight in the role of midgut bacteria in virus infection and transmission. Infection rates were determined for untreated and antibiotic-treated females of the two biting species *C. nubeculosus* and *C. sonorensis*. When comparing *C. nubeculosus* females fed on glucose solution with females fed on glucose solution containing antibiotics, the proportion of SBV infected females significantly increased from 11.2% to 19.6% (χ^2 test, *p*=0.02). For *C. sonorensis,* infection rates increased from 18.2% for untreated to 34.0% for antibiotic-treated females (χ^2 test, *p*=0.14; Fig. 3A & Table S1). The observed increase for *C. sonorensis* was not significant, presumably due to the lower number of replicates for this species. Although the infection rate was higher in antibiotic-treated *C. nubeculosus*, the median virus titer of SBV-infected biting midges was not significantly different between untreated and antibiotic-treated *C. nubeculosus* (Mann-Whitney U test, *p*=0.42), and *C. sonorensis* (Mann-Whitney U test, *p*=0.89; Fig. 3B & Table S1).

Infection rates and transmission efficiencies were determined for *Ae. aegypti* females exposed to infectious blood meals containing ZIKV or CHIKV. No significant differences were found in infection rates between untreated and antibiotic-treated *Ae. aegypti* females exposed to ZIKV (9.0%-12.0%; χ^2 test, *p*=0.64) or CHIKV (90.0%-95.0%; χ^2 test, *p*=0.28; Fig. 3C & Table S1). Moreover, no differences were found between virus titers of bodies of untreated and antibiotic-treated ZIKV-infected females (Mann-Whitney U test, *p*=0.29), or CHIKV-infected females (Mann-Whitney U test, *p*=0.84; Fig. 3D & Table S1).

None of the saliva samples were found positive for ZIKV by CPE, therefore no transmission was observed for any of the ZIKV exposed *Ae. aegypti* females. No significant differences were found in transmission efficiency between untreated and antibiotic-treated *Ae. aegypti* females exposed to CHIKV (χ^2 test, *p*=0.59; Fig. 3E & Table S1). Moreover, virus titers in saliva samples of CHIKV-infected females were all lower than 10^3 $TCID_{50}$/ml (Fig. 3F & Table S1).

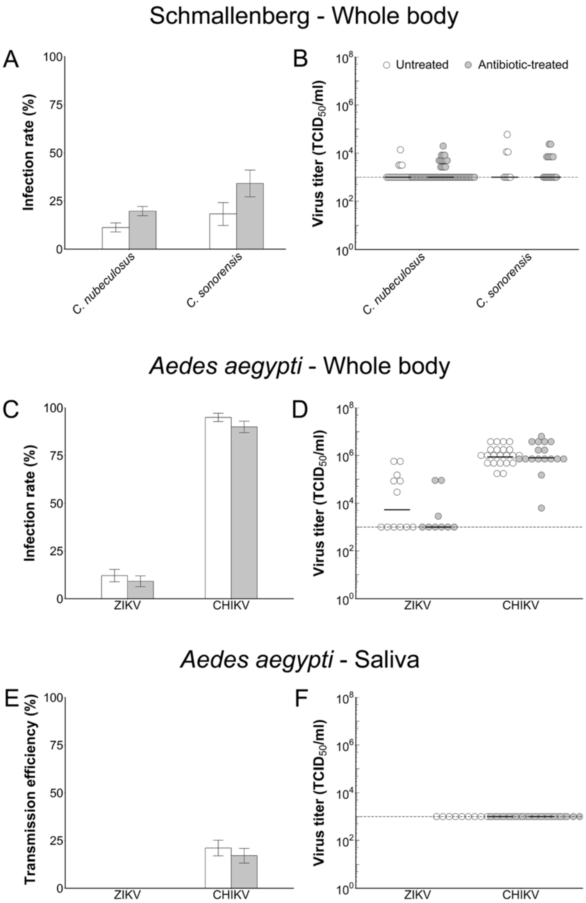

Figure 3. Effect of antibiotic-treatment on susceptibility of biting midges and mosquitoes for arthropod-borne viruses. Panel A: Mean infection rates of Schmallenberg virus (SBV) in biting midges (N_*nubeculosus* = 196, N_*sonorensis* = 44; untreated: white bars) fed on glucose solution, and glucose solution with antibiotics (N_*nubeculosus* = 275, N_*sonorensis* = 47; antibiotic-treated: grey bars). *Culicoides nubeculosus* and *C. sonorensis* were blood-fed three to six days after emergence and tested for virus infection after an incubation period of ten days. Error bars indicate the SEM. Panel B: Average titers of SBV in infected biting midges (*C. nubeculosus* and *C. sonorensis*) for both treatments (untreated: white dots, and antibiotic-treated: grey dots). Each dot represents the titer for one individual biting midge and horizontal bars indicate the median. Panel C: Mean infection rates of Zika virus (ZIKV) and chikungunya virus (CHIKV) in *Aedes aegypti* mosquitoes (N = 100 for each group) fed on glucose solution (untreated: white bars), and glucose solution with antibiotics (antibiotic-treated: grey bars). Mosquitoes were blood-fed four to eight days after emergence and tested for virus infection after an incubation period of ten days. Error bars indicate the SEM. Panel D: Average titer of ZIKV and CHIKV in infected mosquitoes for both treatments (untreated: white dots, and antibiotic-treated: grey dots). Each dot represents the titer for one individual mosquito and horizontal bars indicate the median. Panel E: Percentage positive saliva samples (transmission efficiency) for untreated (white bars) and antibiotic-treated (grey bars) *Ae. aegypti* mosquitoes exposed to ZIKV or CHIKV (N = 100 for each group). Error bars indicate the SEM. Panel F: Average titer of ZIKV and CHIKV positive saliva samples of untreated (white dots) and antibiotic-treated (grey dots) *Ae. aegypti* mosquitoes. No positive saliva samples were found for ZIKV infected mosquitoes. Each dot represents the titer for one individual mosquito and horizontal bars indicate the median.

Discussion

The aim of this study was to investigate if gut bacteria can influence arboviruses infection and transmission in insect vectors. Our data show that feeding insects with antibiotics significantly changed their gut bacterial community composition, which increased virus susceptibility of biting midges, but had no implications for *Ae. aegypti* mosquitoes.

Gut bacterial communities

Antibiotic treatment significantly changed the composition of gut bacterial communities in all three vector species. *Asaia* was identified as the most dominant bacterial genus in gut bacterial communities of the untreated groups, whereas this particular bacterium was almost non-existent in the antibiotic-treated groups. A relative reduction in *Asaia* bacteria therefore may be associated with increased infection of *C. nubeculosus* with SBV. Interestingly, similar changes in the relative abundance of *Asaia* induced by antibiotic-treatment in *Ae. aegypti* did not result in any changes in susceptibility to ZIKV or CHIKV. This suggests that gut bacteria may interact in a specific manner with viruses and their vectors. At this point, we cannot provide conclusive evidence on the effect of *Asaia* on the infectivity of arboviruses in mosquitoes and biting midges. Therefore, we cannot rule out the effect of bacterial gut community density, or of relatively less abundant bacteria on this tripartite interaction. This uncertainty can be illustrated by our findings on gut bacteria of *C. sonorensis*, in which *Asaia* was not the dominant species although we still found a trend towards increased infection in the antibiotic-treated group. Untreated *C. sonorensis* gut bacterial communities were dominated by *Pseudomonas*, Acetobacteraceae, and Azospirillaceae, whereas after treatment with antibiotics communities were dominated by *Sphingomonas* and *Delftia*. These findings point to a potential role of gut bacteria other than *Asaia* in interference with virus infection, or possible effects of overall bacterial density. *Delftia* bacteria were found in antibiotic-treated individuals of both biting midge species, whereas they were not abundant in antibiotic-treated mosquitoes. Re-introduction of specific bacteria such as *Asaia* or *Delftia* in axenic and gnotobiotic biting midges and mosquitoes would provide important insights in species-specific roles in virus-vector interactions (Correa *et al.*, 2018; Guégan *et al.*, 2018; Valzania *et al.*, 2018).

Recently, several studies have shown that bacteria in the gut of laboratory-reared mosquitoes and biting midges are different from those found in field populations (Gimonneau *et al.*, 2014; Muturi *et al.*, 2017; Díaz-Sánchez *et al.*, 2018; Dickson *et al.*, 2018). Therefore, our findings may not directly apply to field populations of mosquitoes and biting midges. While the bacterial communities of laboratory-reared mosquitoes used for this experiment did not show any effect on virus infection or replication, midgut bacterial species found in wild populations may still have an effect. Follow-up studies should focus on identification of bacterial species from field-collected mosquitoes and biting midges (chapter 7), and subsequently test their vector competence.

Virus susceptibility

After antibiotic-treatment, the susceptibility of *C. nubeculosus* to SBV increased, with almost twice as many individuals infected compared to the untreated group. For *C. sonorensis* and *Ae. Aegypti,* infection rates remained equal for SBV and ZIKV and CHIKV, respectively. Moreover, no differences in virus titers were observed between any of the untreated and antibiotic-treated groups. This suggests that the virus replicative fitness remains similar even though infectivity of SBV in the gut of *C. nubeculosus* biting midges is increased after changes in the gut bacterial communities. Absence of a salivary gland barrier for some arboviruses in *Culicoides* biting midges (Fu *et al.*, 1999; Mellor *et al.*, 2000; Mills *et al.*, 2017) suggests that higher infection rates could result in increased vector competence. We, therefore, conclude that exposure of emerging biting midges to antibiotics causes subsequent changes in the gut bacterial communities of biting midges. This could in turn increase the risk for SBV infection of biting midges and subsequent transmission to mammalian hosts.

No effect of antibiotic treatment and consequential changes in the gut bacterial community were found on virus susceptibility or replication for *Ae. aegypti* mosquitoes in our studies. Earlier studies on susceptibility of *Ae. aegypti* for dengue virus (DENV), La Crosse virus, and CHIKV, showed that specific bacteria (i.e. *Serratia odorifera* and *Chromobacterium*) could influence virus replicative fitness inside the mosquito (Apte-Deshpande *et al.*, 2012; Apte-Deshpande *et al.*, 2014; Bourtzis *et al.*, 2014; Ramirez *et al.*, 2014). For instance, the bacteria *Serratia odorifera* positively influenced DENV and CHIKV in *Ae. aegypti* mosquitoes, whereas *Chromobacterium* reduced the infection of DENV in this mosquito species. Here, we identified bacteria from the same families (Enterobacteriaceae and Neisseriaceae), but did not identify bacteria classified as *Serratia* or *Chromobacterium*. The discussed studies found an effect of specific bacteria on virus infection, whereas we did not observe changes in infection after manipulation of the midgut bacterial communities in mosquitoes. This does not necessarily mean that the results of earlier studies and our study are contradictory, but that interactions are likely vector, virus, and bacteria species specific. These results underscore the need to further unravel the complex interactions between midgut bacteria and the infectivity of arboviruses. This will contribute to understanding the possible implications of alterations in midgut bacteria, and how specific bacteria could be used as a novel tool for the control of arboviruses (Mancini *et al.*, 2016; Guégan *et al.*, 2018).

Comparing vector competence of different mosquito or biting midge species, it is evident that some species are better able to transmit viruses than others (chapter 9; Paweska *et al.*, 2002; Turell *et al.*, 2005; Turell *et al.*, 2008; Balenghien *et al.*, 2014; Vogels *et al.*, 2016). This variation in vector competence is shaped by specific interactions between virus, vector, and environmental factors (Hardy *et al.*, 1983; Kenney & Brault, 2014). Our findings support the hypothesis that the gut bacterial community composition of the vector can also, at least in part, explain variation in vector competence (Novakova *et al.*, 2017). Thus, we confirm that midgut bacteria add another level of complexity that should be considered when studying the transmission of arboviruses. Future studies on vector competence of mosquitoes or biting midges should include field-collected individuals, to assess how natural occurring gut bacteria influence their susceptibility to virus infection.

Possible mechanisms

The underlying mechanism of increased susceptibility of *Culicoides* for SBV after antibiotic treatment remains unknown, and will be an important issue for future research. Several possibilities for interaction among midgut bacteria, insect vectors, and pathogens can be considered (Cirimotich, Ramirez*, et al.*, 2011; Hegde *et al.*, 2015). First, the presence of (sufficient) bacteria could be a key factor to reduce virus infection. This could either be through activation of the vector's innate immune responses (Dong *et al.*, 2006; Ryu *et al.*, 2008; Xi *et al.*, 2008; Dong *et al.*, 2009; Barletta *et al.*, 2017), or by directly blocking pathogen interaction with the vector midgut epithelial cells (Azambuja *et al.*, 2005; Kumar *et al.*, 2010; Joyce *et al.*, 2011; Rodgers *et al.*, 2017). Second, direct competition between bacteria and viruses for resources such as lipids or vitamins could affect vector competence (Jupatanakul *et al.*, 2014). Finally, bacterial secretion of specific anti-pathogenic molecules, such as reactive oxygen or secondary metabolites, may kill or interfere with pathogens in the midgut (Cirimotich, Dong, *et al.*, 2011; Joyce *et al.*, 2011; Bahia *et al.*, 2014; Ramirez *et al.*, 2014; Dennison *et al.*, 2016). The presence of *Delftia* in both antibiotic-treated biting midge species suggests they could play a role in facilitation of virus infection. Facilitation of infection was shown for *Anaplasma* bacteria in ticks, where these bacteria enhance cell apoptosis, as well as the production of proteins by the vector that reduce the formation of the peritrophic matrix and biofilms, which in turn resulted in increased infection (Cabezas-Cruz *et al.*, 2016; Abraham *et al.*, 2017).

As mentioned earlier, our findings point to interactions with midgut bacteria that seem specific for each virus-vector combination. It is therefore expected that bacterial species-, or population-specific interactions influence virus infection more than the mere presence of bacteria in the midgut. Bacterial interaction with the vector immune responses or secretion of anti-pathogenic molecules are likely mechanisms for the observed change in infection rates after alteration of the midgut bacteria. Several papers describe the close interaction between bacteria and the innate immune responses of mosquitoes, for example the ability of the microbiota to modulate virus infection through stimulation of the Toll or IMD immune pathway, making this a valuable direction for further research (Xi *et al.*, 2008; Ramirez *et al.*, 2014; Stathopoulos *et al.*, 2014; Barletta *et al.*, 2017).

Although changes in infection rates are likely to be explained by the differences in bacterial communities, an effect of the antibiotic itself on virus-vector interactions cannot be excluded. Antibiotic treatment may inhibit formation of a peritrophic matrix around the blood bolus after blood feeding (Rodgers *et al.*, 2017), thereby enhancing the possibility of virus particles to interact with the midgut epithelial cells. Furthermore, it was shown that antibiotics can induce long-lasting damaging effects on muscle structure and mitochondrial metabolism in blow flies (Renault *et al.*, 2018). Similar effects on midgut cells may result in a "leaky gut", which is a well described physiological change in insect vector midgut cells that results in increased virus infection (Houk *et al.*, 1979; Weaver *et al.*, 1991; Mellor *et al.*, 2000). However, low concentrations used in this study might not be enough to cause physiological damage to the insects tissue. The way in which antibiotics play a role in changing infection rates is unclear, but it can be concluded that the uptake of antibiotics by biting midges results in higher infection rates with SBV. Either through a direct effect of the antibiotic, or through an indirect effect of the antibiotic on microbial communities.

The use of antibiotics in the field

Although the use of antibiotics in the livestock industry has been reduced in several European countries (Cogliani *et al.*, 2011; Speksnijder *et al.*, 2015), the global use of antibiotics consistently increased from the year 2000 to 2015 (Klein *et al.*, 2018). From the antimicrobial compounds used in food production systems, up to 80% ends up in the environment (Sarmah *et al.*, 2006; Andersson & Hughes, 2014). For example, antimicrobial compounds are excreted into the environment by livestock animals via urine or dung, as not all antibiotics are degraded during gut passage. This results in relatively high concentrations of antibiotics in manure (Christian *et al.*, 2003; Martínez-Carballo *et al.*, 2007), and consequently in natural habitats of biting midges and mosquitoes. The use of antibiotics in the livestock industry may, therefore, indirectly affect susceptibility of *Culicoides* vectors for arboviruses, which may result in higher transmission risk of SBV from biting midges to livestock.

Conclusions

Antibiotic uptake and subsequent changes in gut microbial communities, resulted in an almost two-fold higher infection rate of the biting midge species *C. nubeculosus* for SBV. Infection rates in *C. sonorensis* showed a similar trend but were not significantly different between the untreated and antibiotic-treated groups. Use of antimicrobial compounds at livestock farms might therefore have an unexpected contradictory effect on the health of animals, by increasing the transmission of viral pathogens by biting midges. No effect of antibiotic treatment and subsequent shift in bacterial community composition on vector competence of *Ae. aegypti* for ZIKV or CHIKV was detected. We therefore conclude that the effect of midgut bacteria of virus infection is context-dependent and virus-vector specific. Understanding the mechanisms of how (specific) midgut bacteria influence the infectivity of arboviruses in their vectors will contribute to the search for new control strategies for vector-borne diseases.

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Supplementary

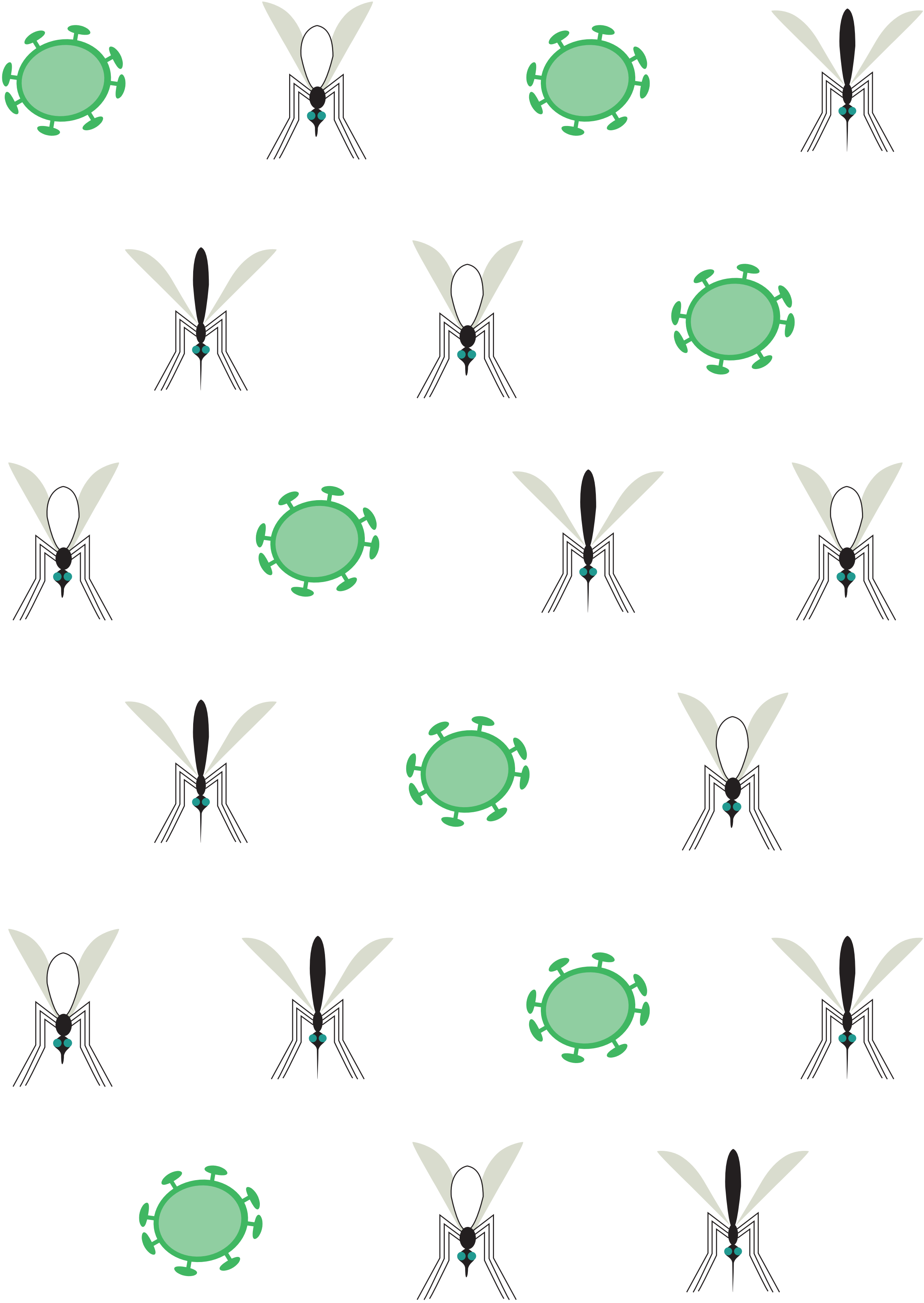
Table S1: Infection rates, transmission efficiencies, and median (ingested) titers of untreated and antibiotic-treated biting midges orally exposed to Schmallenberg virus, and untreated and antibiotic-treated *Aedes aegypti* mosquitoes orally exposed to Zika virus or chikungunya virus. Infection rates and transmission efficiencies were determined as the percentage of insects with virus in their body or saliva, respectively, out of the total number of orally exposed insects within the respective treatment. Infection rates and transmission efficiencies are presented as percentages (number of virus positive bodies or saliva samples / total number of engorged females). Titers were determined for infected biting midge bodies, for mosquitoes infected with ZIKV, and for mosquitoes with a fully disseminated infection of CHIKV. The results represent the cumulative data from three (biting midges) or four (mosquitoes) independent biological replicates.

Species	**Virus**	**Treatment**	**Infection (%)**	**Transmission (%)**	**Median ingested virus titers ($TCID_{50}$/ml)**	**Median titer body ($TCID_{50}$/ml)**	**Median titer saliva ($TCID_{50}$/ml)**
Culicoides nubeculosus	SBV	Untreated	11.2 (22/196)	-	-	1×10^3	-
		Antibiotic	19.6 (54/275)	-	-	1×10^3	-
Culicoides sonorensis		Untreated	18.2 (8/44)	-	-	1×10^3	-
		Antibiotic	34.0 (16/47)	-	-	1×10^3	-
Aedes aegypti	ZIKV	Untreated	12.0 (12/100)	0 (0/100)	1×10^3	1.5×10^4	-
		Antibiotic	9.0 (9/100)	0 (0/100)	1×10^3	1×10^3	-
	CHIKV	Untreated	95.0 (95/100)	21 (21/100)	2×10^5	8.7×10^5	1×10^3
		Antibiotic	90.0 (90/100)	17 (17/100)	1.7×10^5	8.0×10^5	1×10^3

Abbreviations: SBV: Schmallenberg virus, ZIKV: Zika virus, CHIKV: chikungunya virus, untreated: fed with 6% glucose solution, antibiotic: fed with 6% glucose solution with addition of penicillin and streptomycin, $TCID_{50}$/ml: 50% tissue culture infective dose per millilitre.

Table S2. Gut microbial diversity. Estimators of taxonomic diversity for gut microbiota of *Aedes aegypti, Culicoides nubeculosus* and *C. sonorensis* kept on either 6% glucose solution (untreated) or 6% glucose solution with penicillin and streptomycin (antibiotic-treated). Average values (minimum – maximum) are presented for Inverse Simpson Index, Shannon-Wiener Diversity and Shannon-Wiener Evenness.

Taxonomic diversity	Mosquitoes		Biting midges			
	Aedes aegypti		*Culicoides nubeculosus*		*Culicoides sonorensis*	
	Untreated	Antibiotic-treated	Untreated	Antibiotic-treated	Untreated	Antibiotic-treated
No. of samples	12	12	18	15	10	9
Inverse Simpson Index	**1.501** (1.009 – 3.063)	**3.038** (1.031 – 11.702)	**1.257** (1.011 – 2.070)	**2.915** (1.237 – 5.901)	**2.437** (1.074 – 7.647)	**3.135** (1.014 – 8.401)
Shannon-Wiener diversity	**0.527** (0.039 – 1.720)	**1.055** (0.114 – 3.009)	**0.351** (0.046 – 0.837)	**1.339** (0.528 – 2.150)	**1.070** (0.202 – 2.771)	**1.265** (0.061 – 3.289)
Shannon-Wiener evenness	**1.236** (1.031 – 1.823)	**1.385** (1.087 – 1.940)	**1.158** (1.036 – 1.332)	**1.447** (1.302 – 1.730)	**1.522** (1.139 – 2.090)	**1.468** (1.048 – 3.192)



Chapter 9

Vector competence of biting midges and mosquitoes for Shuni virus

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Abstract

Background: Shuni virus (SHUV) is an orthobunyavirus that belongs to the Simbu serogroup. SHUV was isolated from diverse species of domesticated animals and wildlife, and is associated with neurological disease, abortions, and congenital malformations. Recently, SHUV caused outbreaks among ruminants in Israel, representing the first incursions outside the African continent. The isolation of SHUV from a febrile child in Nigeria and seroprevalence among veterinarians in South Africa suggests that the virus may have zoonotic potential as well. The high pathogenicity, extremely broad tropism, potential transmission via both biting midges and mosquitoes, and zoonotic features warrants prioritization of SHUV for further research. Additional knowledge is essential to accurately determine the risk for animal and human health, and to assess the risk of future epizootics and epidemics. To gain first insights into the potential involvement of arthropod vectors in SHUV transmission, we have investigated the ability of SHUV to infect and disseminate in laboratory-reared biting midges and mosquitoes. **Methodology/Principal Findings**: *Culicoides nubeculosus, C. sonorensis, Culex pipiens pipiens*, and *Aedes aegypti* were orally exposed to SHUV by providing an infectious blood meal. Biting midges showed high infection rates of approximately 40-60%, whereas infection rates of mosquitoes were lower than 2%. SHUV successfully disseminated in both species of biting midges, but no evidence of transmission in orally exposed mosquitoes was found. **Conclusions/Significance**: The results of this study show that different species of *Culicoides* biting midges are susceptible to infection and dissemination of SHUV, whereas the two mosquito species tested were found not to be susceptible.

Keywords: Shuni virus, *Culicoides nubeculosus*, *Culicoides sonorensis*, *Culex pipien*s, *Aedes aegypti*, emerging disease, livestock pathogen, zoonosis, infection, dissemination

Author summary

Arthropod-borne (arbo)viruses are notorious for causing unpredictable and large-scale epidemics and epizootics. Apart from viruses such as West Nile virus and Rift Valley fever virus that are well known to have a significant impact on human and animal health, many arboviruses remain neglected. Shuni virus (SHUV) is a neglected virus with zoonotic potential that was recently associated with severe disease in livestock and wildlife. Isolations of SHUV from field-collected biting midges and mosquitoes suggests that SHUV may be transmitted by these insects. Laboratory-reared biting midge species (*Culicoides nubeculosus* and *C. sonorensis*) and mosquito species (*Culex pipiens pipiens* and *Aedes aegypti*), that are known to transmit other arboviruses, were exposed to SHUV via an infectious blood meal. SHUV was able to successfully disseminate in both biting midge species, whereas no evidence of infection or transmission in both mosquito species was found. Our results show that SHUV infects and disseminates in two different *Culicoides* species, suggesting that these insects could play an important role in the disease transmission cycle.

Introduction

Arthropod-borne (arbo)viruses continue to pose a threat to human and animal health (Gubler, 2002; Mayer *et al.*, 2017). In particular the order *Bunyavirales* comprises emerging pathogens such as Crimean-Congo haemorrhagic fever virus (CCHFV) and Rift Valley fever virus (RVFV) (Ergönül, 2006; Bird *et al.*, 2009). The World Health Organization (WHO) has included both CCHFV and RVFV to the "Blueprint" list of ten prioritized viruses likely to cause future epidemics and for which insufficient countermeasures are available (World Health Organization, 2018). In the veterinary field, prioritized viral diseases of animals, including RVFV, are notifiable to the World Organization for Animal Health (Office International des Epizooties, OIE). Apart from pathogens that are recognised as major threats by WHO and OIE, many have remained largely neglected. Before the turn of the century, West Nile virus, chikungunya virus, and Zika virus were among these neglected viruses until they reminded us how fast arboviruses can spread in immunologically naïve populations (Mayer *et al.*, 2017). Although these outbreaks came as a surprise, in hindsight, smaller outbreaks in previously unaffected areas could have been recognised as warning signs.

Shuni virus (SHUV; family *Peribunyaviridae*, genus *Orthobunyavirus*, Simbu serogroup) recently emerged in two very distant areas of the world (van Eeden *et al.*, 2014). SHUV was isolated for the first time from a slaughtered cow in the 1960s in Nigeria (Causey *et al.*, 1972). During subsequent years, the virus was isolated on several occasions from domestic animals including cattle, sheep, goats, and horses (Causey *et al.*, 1972; Coetzer & Erasmus, 1994; Venter *et al.*, 2010; van Eeden *et al.*, 2012), from wild animals including crocodiles and rhinoceros (Venter *et al.*, 2010), and from field-collected *Culicoides* biting midges and mosquitoes (McIntosh *et al.*, 1972; Lee, 1979; Coetzer & Erasmus, 1994). More recently, SHUV was associated with malformed ruminants in Israel (Golender *et al.*, 2015; Golender *et al.*, 2016). Emergence of SHUV in areas outside Sub-Saharan Africa shows the potential of this virus to spread to new areas, and increases the risk for SHUV outbreaks in bordering territories such as Europe. Isolation of SHUV from a febrile child and detection of antibodies in 3.9% of serum samples from veterinarians in South Africa shows that SHUV can infect humans as well, although its ability to cause human disease is still uncertain (Causey *et al.*, 1972; Moore *et al.*, 1975; van Eeden, Swanepoel, *et al.*, 2014).

Proper risk assessments rely on accurate knowledge of disease transmission cycles. Arbovirus transmission cycles can only become established when competent vectors and susceptible hosts encounter under suitable climatic conditions. Although SHUV has been isolated from pools of field-collected *Culicoides* biting midges and mosquitoes (Causey *et al.*, 1972; McIntosh *et al.*, 1972; Lee, 1979), the role of both insect groups as actual vectors remains to be confirmed. Detection of virus in field-collected insects is not sufficient to prove their ability to transmit the virus. Arboviruses need to overcome several barriers (*i.e.* midgut and salivary gland barriers) inside their vector, before they can be transmitted (Mills *et al.*, 2017; Vogels *et al.*, 2017a). In addition to virus isolation from field-collected vectors, laboratory studies are therefore needed to experimentally test the ability of blood-feeding insects to become infected with, maintain, and successfully transmit arboviruses (*i.e.*, vector competence) (Kenney & Brault, 2014). To gain insights into the potential of *Culicoides* biting midges and mosquitoes to function as vectors of SHUV, we studied the susceptibility of four main arbovirus vector species (*Culicoides nubeculosus* and *C. sonorensis* biting midges, and *Culex pipiens* biotype *pipiens* and *Aedes aegypti* mosquitoes) for SHUV.

Materials and Methods

Cell culture

African green monkey kidney cells (Vero E6; ATCC CRL-1586) were cultured in Eagle's minimum essential medium (Gibco, Carlsbad, CA, United States) supplemented with 5% fetal bovine serum (FBS; Gibco), 1% non-essential amino acids (Gibco), 1% L-glutamine (Gibco), and 1% antibiotic/antimycotic (Gibco). Cells were cultured as monolayers and maintained at 37°C with 5% CO_2.

Vero E6 cells that were used in biting midge and mosquito infection experiments in the biosafety level 3 (BSL3) facility were cultured in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% FBS, penicillin (100 U/ml; Sigma-Aldrich, Saint Louis, MO, United States), and streptomycin (100 µg/ml; Sigma-Aldrich). Prior to infections in the BSL3 facility, Vero E6 cells were seeded in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered DMEM medium (HEPES-DMEM; Gibco) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml), fungizone (50 µg/ml; Invitrogen, Carlsbad, United States), and gentamycin (50 µg/ml; Gibco).

C6/36 cells (ATCC CRL-1660), derived from *Ae. albopictus* mosquitoes, were cultured in Leibovitz-15 (L-15) growth medium (Sigma-Aldrich) supplemented with 10% FBS, 2% Tryptose Phosphate Broth (Gibco), 1% non-essential amino acids solution, and 1% antibiotic/antimycotic. Cells were cultured as monolayers and incubated at 28°C in absence of CO_2.

KC cells, derived from embryos of colonized *C. sonorensis* biting midges (Wechsler *et al.*, 1989), were cultured as monolayers in modified Schneider's *Drosophila* medium (Lonza, Basel, Switzerland) with 15% FBS, and 1% antibiotic/antimycotic at 28°C in absence of CO_2.

Virus

SHUV (strain An10107, P2 Vero, 1980) was kindly provided by the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA). The virus was originally isolated from the blood of a slaughtered cow in 1966 in Nigeria by inoculation of neonatal mice, and passaged twice in Vero cells (Centers for Disease Control and Prevention). The passage 3 (P3) stock was generated by inoculation of Vero E6 cells with the P2 stock at a multiplicity of infection (MOI) of 0.001. The supernatant was harvested at 6 days post inoculation, centrifuged, and stored in aliquots at -80°C. The P4 stock was generated by inoculating Vero E6 cells at MOI 0.01 using the P3 stock. At this MOI, full cytopathic effect (CPE) was present at 3 days post infection. Virus titers were determined using endpoint dilution assays (EPDA) on Vero E6 cells (Vloet *et al.*, 2017). Titers were calculated using the Spearman-Kärber algorithm and expressed as 50% tissue culture infective dose ($TCID_{50}$) (Spearman, 1908; Kärber, 1931). The virus detection and titration procedure was validated using a SHUV-specific reverse transcriptase quantitative PCR (RT-qPCR; S1 Supporting Information).

Growth curves

Cells were seeded in T25 cell culture flasks at densities of 7.5×10^5 (Vero E6), 1.5×10^6 (C6/36), or 2.5×10^6 (KC cells) per flask in 10 ml complete medium. After overnight incubation, the flasks were inoculated with SHUV at an MOI of 0.01 (P4 stock). The MOI calculation for each cell line was based on the virus titer that was determined on Vero E6 cells. One hour after inoculation, the medium was removed and replaced with fresh medium. At time points 0 (sample taken directly after medium replacement), 24, 48, and 72 h post infection, 200 µl samples were taken and stored at -80°C for later analysis. For each cell line, virus titers were determined in triplicate per time point by EPDA using Vero E6 cells, which showed distinct CPE (Vloet *et al.*, 2017).

Insect rearing

Culicoides nubeculosus were kindly provided by The Pirbright Institute, Pirbright laboratories, United Kingdom, in 2012 (Boorman, 1974), and were maintained at 23°C with 16:8 light:dark cycle and 60% relative humidity. *Culicoides sonorensis* were kindly provided by the Arthropod-Borne Animal Diseases Research Laboratory, USDA-ARS (courtesy of Dr. Barbara Drolet) in 2017 (Nayduch *et al.*, 2014)2014, and were maintained at 25°C with 16:8 light:dark cycle and 70% relative humidity. Similar rearing protocols were used for both biting midge species. Eggs were transferred to square larval holding trays (*C. nubeculosus*: 25 x 25 x 8 cm, Kartell, Noviglio, Italy; *C. sonorensis:* 19 x 19 x 20 cm, Jokey, Wipperfürth, Germany) with filter wool (Europet Bernina International, Gemert-Bakel, The Netherlands) attached with double-sided tape to the bottom. Trays were filled with tap water, a few millilitres of rearing water in which larvae had completed their life cycle, and two drops of Liquifry No.1 (Interpet, Dorking, United Kingdom). Larvae were fed with a 1:1:1 mixture of bovine liver powder (MP biomedicals, Irvine, CA, US), ground rabbit food (Pets Place, Ede, The Netherlands), and ground koi food (Tetra, Melle, Germany). *Culicoides nubeculosus* larvae were additionally fed with nutrient broth No. 2 (Oxoid, Hampshire, UK). Pupae were transferred to plastic buckets (diameter: 12.2 cm, height: 12.2 cm; Jokey) and closed with netting on the top through which the biting midges could feed. Emerged adults were provided with 6% glucose solution *ad libitum*. Cow blood (Carus, Wageningen, The Netherlands) was provided through a Parafilm M membrane using the Hemotek PS5 feeding system (Discovery Workshops, Lancashire, United Kingdom) for egg production.

The *Cx. pipiens pipiens* colony was established in the laboratory from egg rafts collected in the field in The Netherlands during August 2016. Egg rafts were individually hatched in tubes. Pools of approximately 10 first instar larvae were identified to the biotype level using real-time PCR (Vogels *et al.*, 2015). The colony was started by grouping larvae from 93 egg rafts identified as the *pipiens* biotype. Mosquitoes were maintained at 23°C with 16:8 light:dark cycle and 60% relative humidity (Vogels *et al.*, 2016; Vogels *et al.*, 2017b). Adult mosquitoes were kept in Bugdorm-1 rearing cages and maintained on 6% glucose solution *ad libitum*. Cow blood or chicken blood (Kemperkip, Uden, The Netherlands) was collected in BC Vacutainer lithium heparin-coated blood collection tubes (Becton Dickinson, Breda, The Netherlands), and stored at 4°C. Blood was provided through a Parafilm M membrane using the Hemotek PS5 feeding system for egg production. Egg rafts were transferred to square larval holding trays (25 x 25 x 8 cm, Kartell) filled with tap water and two drops of Liquifry No. 1. Hatched larvae were fed with a 1:1:1 mixture of bovine liver powder, ground rabbit food, and ground koi food. Pupae were collected every 2 days and placed in Bugdorm-1 insect rearing cages.

Aedes aegypti mosquitoes from the Rockefeller strain (Bayer AG, Monheim, Germany) were used in all experiments. The mosquito colony was maintained as described before (Göertz *et al.*, 2017). In short, mosquitoes were maintained at 27°C with 12:12 light:dark cycle and 70% relative humidity. Adult mosquitoes were kept in Bugdorm-1 rearing cages and maintained on 6% glucose solution *ad libitum*. Human blood (Sanquin Blood Supply Foundation, Nijmegen, The Netherlands) was provided through a Parafilm M membrane using the Hemotek PS5 feeding system for egg production. Eggs were transferred to transparent square larval holding trays (19 x 19 x 20 cm, Jokey), filled for approximately one-third with tap water and three drops of Liquifry No. 1. Hatched larvae were fed with Tetramin Baby fish food (Tetra). Larval trays were closed with fine-meshed netting, to allow adult mosquitoes to emerge inside larval trays. Twice a week, adults were aspirated from the larval trays and collected in Bugdorm-1 insect rearing cages.

Feeding of biting midges and mosquitoes with SHUV infectious blood

Groups of adult *C. nubeculosus* (1-7 days old), *C. sonorensis* (1-11 days old), *Cx. p. pipiens* (4-20 days old), and *Ae. aegypti* (4-7 days old) were transferred to plastic buckets (diameter: 12.2 cm, height: 12.2 cm; Jokey) and closed with netting before being taken to the BSL3 facility. *Culex p. pipiens* mosquitoes were kept on water for 3 days, whereas the other species were maintained on 6% glucose solution until being offered an infectious blood meal. SHUV P3 stock with a mean titer of 3.0 x 10^6 $TCID_{50}$/ml was mixed 1:1 with cow blood. The used cow blood was tested negative for Schmallenberg virus (SBV) antibodies, to prevent cross-neutralisation with SHUV. The infectious blood meal was provided through a Parafilm M membrane using the Hemotek PS5 feeding system, under dark conditions at 24°C and 70% relative humidity.

After 1 h, insects were anesthetized with 100% CO_2 and kept on a CO_2-pad to select fully engorged females. For each species, five fully engorged females were directly stored at -80°C for each replicate. These samples were used to determine the ingested amounts of SHUV for each species. All remaining and fully engorged females were placed back into buckets with a maximum group size of 110 individuals per species per bucket. All insects were provided with 6% glucose solution via a soaked ball of cotton wool on top of the netting *ad libitum*. *Culicoides sonorensis* and *Ae. aegypti* were kept at 28°C for 10 days, whereas *C. nubeculosus* and *Cx. p. pipiens* were kept at 25°C for 10 days. These temperatures were selected for optimal replication of the virus, and to reflect differences in the rearing temperature for each species. Three replicate experiments of *C. nubeculosus* ($N_1 = 84$, $N_2 = 82$, $N_3 = 77$, $N_{total} = 243$), *C. sonorensis* ($N_1 = 9$, $N_2 = 9$, $N_3 = 30$, $N_{total} = 48$), and *Cx. p. pipiens* ($N_1 = 89$, $N_2 = 57$, $N_3 = 65$, $N_{total} = 211$) were carried out, and two replicate experiments of *Ae. aegypti* ($N_1 = 72$, $N_2 = 77$, $N_{total} = 149$). During each replicate, biting midges and mosquitoes were fed in parallel with the same infectious blood meal.

Intrathoracic injections of mosquitoes with SHUV

Adult female *Cx. p. pipiens* (3-9 days old) and *Ae. aegypti* (4-6 days old) mosquitoes were injected with SHUV into the thorax to investigate the role of mosquito barriers on dissemination of SHUV. Mosquitoes were anesthetized with 100% CO_2 and positioned on the CO_2-pad. Female mosquitoes were intrathoracically injected with 69 nl of SHUV (P3 stock with a titer of 3.0×10^6 $TCID_{50}$/ml) using a Drummond Nanoject II Auto-Nanoliter injector (Drummond Scientific, Broomall, Unites States). Injected *Cx. p. pipiens* were maintained at 25°C and injected *Ae. aegypti* were maintained at 28°C. Mosquitoes were incubated for 10 days at the respective temperatures, and had access to 6% glucose solution *ad libitum*. Injections were done during a single replicate experiment for *Cx. p. pipiens* (N = 50) and *Ae. aegypti* (N = 50).

Infectivity assays

After 10 days of incubation at the respective incubation temperatures, samples from surviving biting midges and mosquitoes were collected. Biting midges were anesthetized with 100% CO_2 and transferred individually to 1.5 ml Safe-Seal micro tubes (Sarstedt, Nümbrecht, Germany) containing 0.5 mm zirconium beads (Next Advance, Averill Park, NY, United States). For a selection of *C. nubeculosus* (N = 77) and *C. sonorensis* (N = 30) from one replicate experiment, heads were removed from bodies and separately stored in tubes. All samples were stored at -80°C until further processing.

Mosquitoes were anesthetized with 100% CO_2 to remove legs and wings. Mosquito saliva was then collected by inserting the proboscis into a 200 µl yellow pipette tip (Greiner Bio-One) containing 5 µl of a 1:1 solution of 50% glucose solution and FBS. The saliva sample was transferred to a 1.5 ml micro tube containing 55 µl of fully supplemented HEPES-DMEM medium. Mosquito bodies were individually stored in 1.5 ml Safe-Seal micro tubes containing 0.5 mm zirconium beads.

Frozen biting midge and mosquito tissues were homogenized for 2 min at maximum speed (setting 10) in the Bullet Blender Storm (Next advance), centrifuged for 30 seconds at 14,500 rpm in the Eppendorf minispin plus (Eppendorf, Hamburg, Germany), and suspended in 100 µl of fully supplemented HEPES-DMEM medium. After addition of the medium, samples were blended again for 2 min at maximum speed, and centrifuged for 2 min at 14,500 rpm. Mosquito saliva samples were thawed at RT and vortexed before further use. In total 30 µl of each body or saliva sample was inoculated on a monolayer of Vero E6 cells in a 96 wells plate. SHUV stock or infectious blood mixture was included as positive control and wells to which no sample was added were included as negative controls. After 2-3 h the inoculum was removed and replaced by 100 µl of fully supplemented HEPES-DMEM medium. Wells were scored for virus induced CPE at 3 and 7 days post inoculation, with full CPE being observed at the latter time point. Afterwards, virus titers for positive samples of biting midge bodies and heads, as well as mosquito bodies and saliva were determined with single EPDA on Vero E6 cells (Göertz *et al.*, 2017). Virus titers were determined using the Reed & Muench algorithm (Reed & Muench, 1938). A subset of samples was validated by RT-qPCR, to confirm that observed CPE was induced by SHUV (S1 Supporting Information).

Infection rate (virus-infected whole body) and dissemination efficiency (virus-infected head) were determined for biting midges, whereas infection rate (virus-infected whole body) and transmission efficiency (virus-infected saliva) were determined for mosquitoes. Infection rate, dissemination efficiency, and transmission efficiency were calculated, respectively, by dividing the number of females with virus-infected bodies (infection), virus-infected heads (dissemination), or virus-infected saliva (transmission) by the total number of females tested in the respective treatment and that survived the incubation period. The values were subsequently expressed as percentages by multiplying with 100. Two biting midge samples of which only the head was virus-positive, but not the body, were considered to be uninfected.

Results

Efficient growth of SHUV in mammalian, mosquito, and midge cells

Mammalian, mosquito, and midge cells were inoculated with SHUV to gain insight into the replicative fitness of this virus and strain in different host cell types. The results show that SHUV is capable to produce progeny in all three cell types (Fig. 1 and S1. Data). Of note, a strong CPE was observed in the Vero E6 cells upon infection whereas no CPE was observed in the insect cell lines. Therefore, Vero E6 cells were used to determine titers by EPDA.

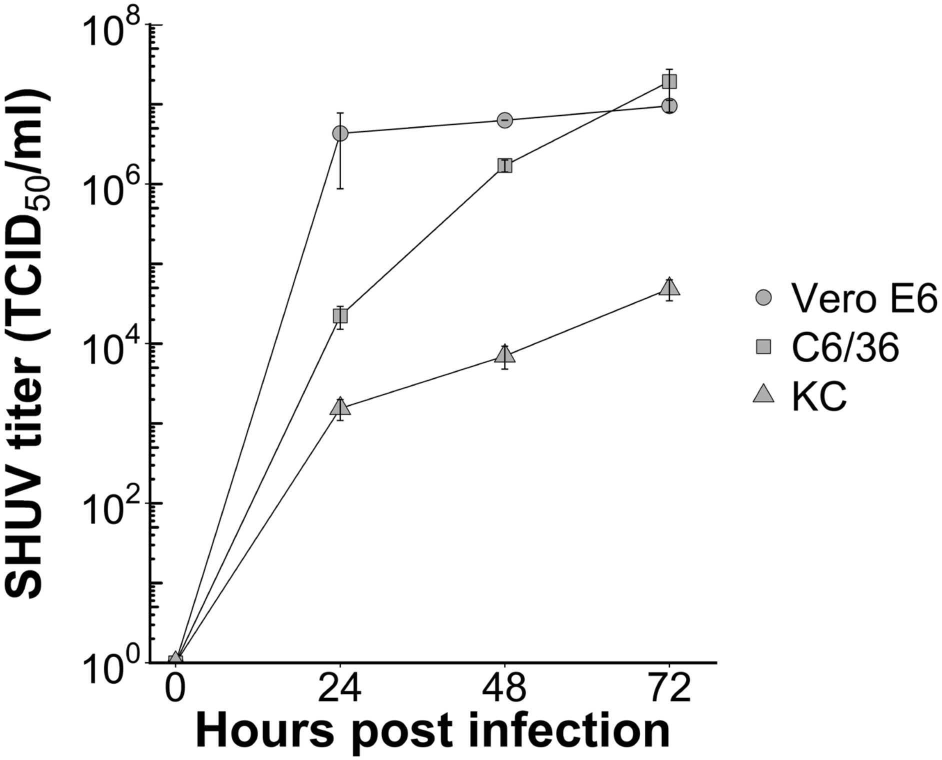

Figure 1. Growth of Shuni virus (SHUV) in mammalian (Vero E6), mosquito (C6/36), and *Culicoides* biting midge (KC) cells. Three different cell lines (African green monkey (Vero E6) cells, *Aedes albopictus* (C6/36) cells, and *Culicoides sonorensis* (KC) cells) were inoculated with SHUV at an MOI of 0.01, and kept at 28°C (C6/36 and KC) or 37°C (Vero E6). Virus titers were determined at time points 0, 24, 48, and 72 h post infection. Mean virus titers ± SEM for three replicates are shown.

Culicoides biting midges are highly susceptible to SHUV infection

To evaluate the susceptibility of two species of biting midges (*C. nubeculosus* and *C. sonorensis*) for SHUV, groups of individuals of both species were orally exposed to an infectious blood meal with a mean SHUV titer of 3.0 x 10^6 $TCID_{50}$/ml. SHUV titers of ingested blood were determined for a selection of 10 fully engorged females for each species, that were directly stored at -80°C after feeding. Both species ingested low amounts of SHUV that were below the detection limit of the endpoint dilution assay of 10^3 $TCID_{50}$/ml.

Infection rates were also determined after 10 days of incubation at temperatures of 25°C (*C. nubeculosus* and *Cx. p. pipiens*) or 28°C (*C. sonorensis* and *Ae. aegypti;* Fig. 2 and S2. Data). Both biting midge species showed high infection rates of 44% for *C. nubeculosus* (N = 243), and 60% for *C. sonorensis* (N = 48; Fig. 2A). SHUV replicated to median titers of 2.4 x 10^3 $TCID_{50}$/ml in body samples of *C. nubeculosus* and 1.1 x 10^4 $TCID_{50}$/ml in body samples of *C. sonorensis* (Fig. 2E). For one replicate experiment, heads were separated from the bodies and tested for presence of SHUV to assess whether the virus successfully passed from the midgut to the haemocoel, indicative of dissemination throughout the body. Dissemination efficiencies were 18% (N = 77) for *C. nubeculosus* and 10% (N = 30) for *C. sonorensis* (Fig. 2C). In all virus-positive heads that induced CPE, SHUV titers were lower than 10^3 $TCID_{50}$/ml. Because only very low amounts of SHUV were detected in biting midge heads, the actual percentage of disseminated infections might be higher. A subset of the samples was additionally tested by RT-qPCR to confirm that CPE was induced by SHUV (S1 Supporting Information). The relatively high infection rates and dissemination efficiencies observed in this study and the absence of a salivary glands barrier in biting midges as shown in previous studies (Fu *et al.*, 1999; Mills *et al.*, 2017), suggests that both *C. nubeculosus* and *C. sonorensis* have the potential to transmit SHUV.

Low susceptibility of mosquitoes to SHUV

SHUV was previously isolated from field-collected mosquitoes (Coetzer & Erasmus, 1994). Therefore, we determined vector competence for two mosquito species (*Cx. p. pipiens* and *Ae. aegypti*) which are important vectors for several arboviruses (Vogels *et al.*, 2016; Göertz *et al.*, 2017; Vloet *et al.*, 2017). SHUV titers of ingested blood were determined for a selection of 10 fully engorged female mosquitoes that were directly stored at -80°C after feeding on an infectious blood meal with a SHUV titer of 3.0 x 10^6 $TCID_{50}$/ml. Similar to results obtained with the biting midges, the amounts of SHUV ingested by both mosquito species was less than 10^3 $TCID_{50}$/ml.

No SHUV infection was observed in the *Cx. p. pipiens* mosquitoes (N = 211) following oral exposure, whereas infection rates of 2% were found for orally exposed *Ae. aegypti* mosquitoes (N = 149; Fig. 2B). SHUV replicated to median titers of 6.3 x 10^3 $TCID_{50}$/ml in body samples of *Ae. aegypti* (Fig. 2F), which was comparable to titers found in biting midges. No SHUV was

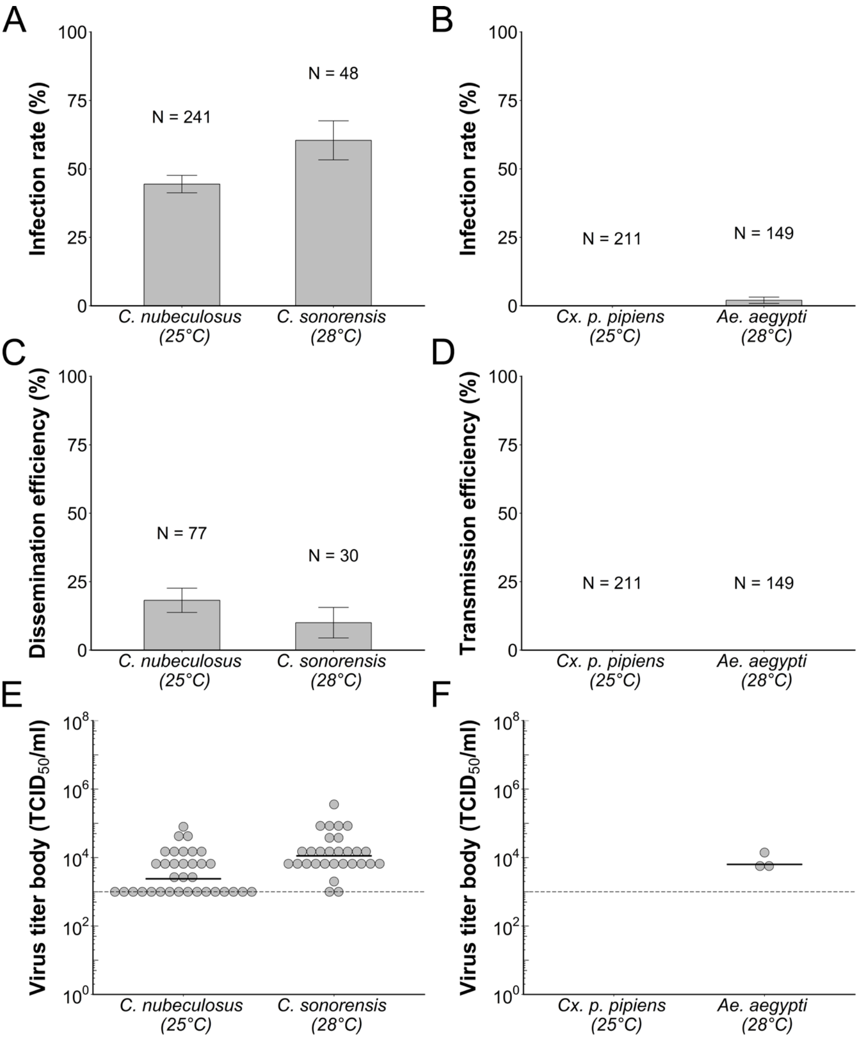

Figure 2. Susceptibility of orally exposed biting midges and mosquitoes for Shuni virus (SHUV). Mean infection rates of *Culicoides nubeculosus* (A; N = 243, 25°C), *C. sonorensis* (A; N = 48, 28°C), *Culex pipiens pipiens* (B; N = 211, 25°C), and *Aedes aegypti* (B; N = 149, 28°C) orally exposed to SHUV after 10 days of incubation at the respective temperatures. Infection rate represents the percentage of virus-positive females out of the total number of blood-fed females that remained alive at the end of the incubation period. Error bars indicate the SEM. Mean dissemination efficiency (C) of *C. nubeculosus* (N = 77, 25°C) and *C. sonorensis* (N = 30, 28°C). Dissemination efficiency represents the percentage of biting midge females with virus-positive heads out of the total number of blood-fed female biting midges that were alive at the end of the incubation period. Mean transmission efficiency (D) of *Cx. p. pipiens* (N = 211, 25°C) and *Ae. aegypti* mosquitoes (N = 149, 28°C). Transmission efficiency represents the percentage of female mosquitoes with virus-positive saliva out of the total number of blood-fed female mosquitoes that were alive at the end of the incubation period. Error bars indicate the SEM. SHUV titers of virus-positive bodies of *C. nubeculosus* (E; N = 34, 25°C), *C. sonorensis* (E; N = 29, 28°C), and *Ae. aegypti* (F; N = 3, 28°C) after 10 days incubation at the respective temperatures. Each dot represents one individual female, and the black bar indicates the median. The detection limit of the endpoint dilution assay is indicated with the dashed line.

detected in any of the saliva samples taken from either *Cx. p. pipiens* or *Ae. aegypti* (Fig. 2D). Thus, SHUV was able to successfully infect a small proportion of *Ae. aegypti* mosquitoes but not *Cx. p. pipiens*, and no evidence was found for transmission of SHUV by mosquitoes.

The very low infection rates of mosquitoes triggered further investigation into potential mosquito barriers against SHUV infection. To this end, *Cx. p. pipiens* and *Ae. aegypti* mosquitoes were intrathoracically injected with SHUV, to bypass the potential midgut barrier. Direct injection of SHUV into the thorax resulted in high infection rates of 70% for *Cx. p. pipiens* (N = 50), and 100% for *Ae. aegypti* (N = 50; Fig. 3A). Transmission efficiency of 32% (N = 50) was found for *Cx. p. pipiens* and 8% (N = 50) for *Ae. aegypti* (Fig. 3B). Interestingly, although infection rates of *Cx. p. pipiens* were below 100%, we found a relatively high transmission efficiency. This may indicate a relatively weaker salivary gland barrier in *Cx. p. pipiens* compared to *Ae. aegypti* mosquitoes that had 100% infection rate, but relatively low transmission efficiency.

To gain more insight in replication of SHUV, virus titers were determined for virus-infected mosquito body and saliva samples. Titers of virus-infected *Cx. p. pipiens* body samples were almost all below the detection limit of 10^3 $TCID_{50}$/ml of the endpoint dilution assay (Fig. 3C). This indicates that even when SHUV is injected into the thorax, there is no productive virus replication. In contrast, we found median titers of 7.1 x 10^4 $TCID_{50}$/ml for virus-infected *Ae. aegypti* body samples. This shows that SHUV is able to successfully replicate in *Ae. aegypti* when the midgut barrier is bypassed. In the majority of mosquito saliva samples, SHUV titers were less than 10^3 $TCID_{50}$/ml (Fig. 3D). Taken together, SHUV is able to disseminate in mosquitoes, but both the midgut and salivary glands form a barrier for SHUV.

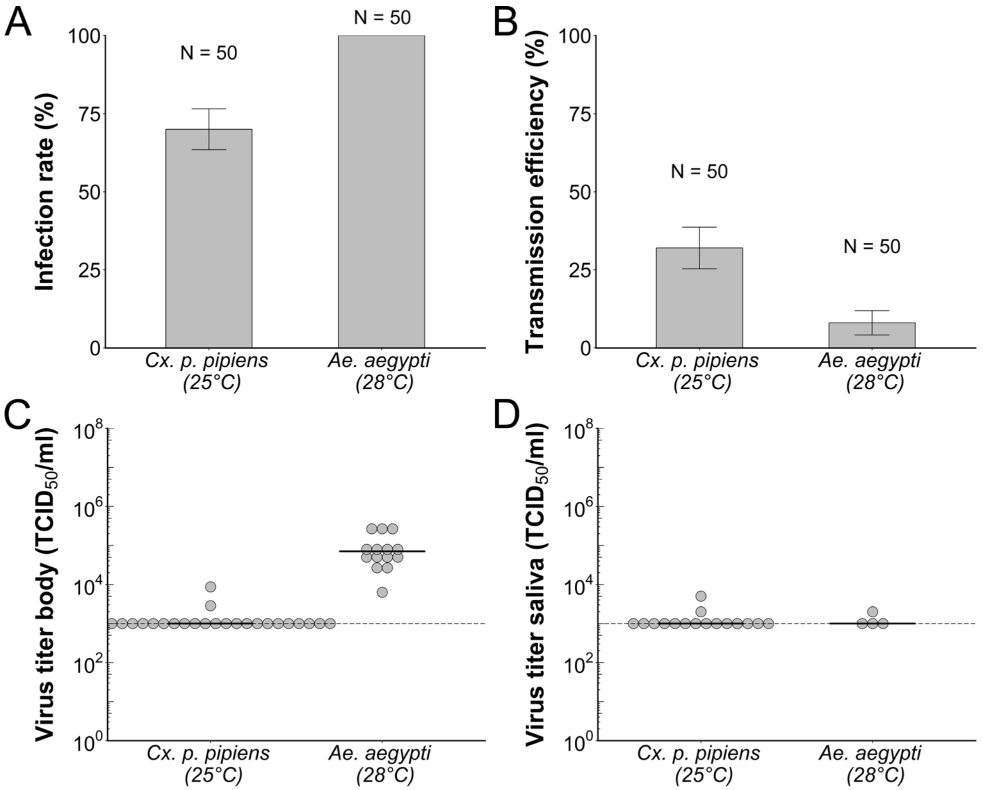

Figure 3. Susceptibility of intrathoracically injected mosquitoes for Shuni virus (SHUV). Mean infection rates (A) and transmission efficiencies (B) of *Culex pipiens pipiens* (N = 50) and *Aedes aegypti* (N = 50) intrathoracically injected with SHUV after 10 days incubation at 25°C and 28°C, respectively. Infection rate represents the percentage of females with virus-positive bodies out of the total number of injected female mosquitoes that were alive at the end of the incubation period. Transmission efficiency represents the percentage of females with virus-positive saliva out of the total number of injected female mosquitoes that were alive at the end of the incubation period. Error bars indicate the SEM as calculated for technical replicates. SHUV titers of virus-positive body samples (C) and saliva samples (D) of *Cx. p. pipiens* (body samples: N = 26, saliva samples: N = 16) and *Ae. aegypti* (body samples: N = 14, saliva samples: N = 4) intrathoracically injected with SHUV after 10 days incubation at 25°C and 28°C, respectively. Each dot represents an individual sample, and the black bar indicates the median. The detection limit of the endpoint dilution assay is indicated with the dashed line.

Discussion

SHUV was previously isolated from field-collected pools of *Culicoides* biting midges and from mosquitoes, but their involvement in SHUV transmission remained to be confirmed (McIntosh *et al.*, 1972; Lee, 1979; Coetzer & Erasmus, 1994). Here, we show for the first time that SHUV is able to infect and replicate in biting midges as well as in mosquitoes, but only the biting midge species evaluated in the present study can be considered highly susceptible to infection.

Both *C. nubeculosus* and *C. sonorensis* showed high infection rates of 44% and 60% when incubated for 10 days at 25°C and 28°C, respectively. It has been demonstrated that a salivary gland barrier is absent for Orbiviruses and Schmallenberg virus in biting midges (Fu *et al.*, 1999; Mills *et al.*, 2017). This knowledge, in combination with evidence of successful dissemination of SHUV to the heads indicates that the biting midge species evaluated in the present study are likely competent vectors of SHUV. Importantly, the finding that SHUV replicates efficiently in two biting midge species from a different geographic background suggests that various species of *Culicoides* may function as vectors of SHUV.

SHUV infection and replication in biting midges seems more efficient compared to other biting midge-borne viruses such as SBV and bluetongue virus (BTV), which generally show infection rates up to 30% (Fu *et al.*, 1999; Carpenter *et al.*, 2006; Veronesi, Antony, *et al.*, 2013; Veronesi, Henstock, *et al.*, 2013; Barber *et al.*, 2018). Both SBV and BTV have caused sudden and large-scale epizootics in Europe, with devastating consequences for the livestock sector (Saegerman *et al.*, 2008; Beer *et al.*, 2013). The relatively high susceptibility and efficiency of replication in biting midges, and recent spread of SHUV to areas outside Sub-Saharan Africa (Golender *et al.*, 2015), should therefore be interpreted as a warning for its epizootic potential.

In contrast to the high infection rates in biting midges, only few orally exposed *Ae. aegypti* mosquitoes became infected with SHUV during 10 days of incubation at 28°C. In addition, no evidence of successful dissemination to the salivary glands of the two mosquito species was found. SHUV replication and transmission (8%) was observed when the virus was directly injected into the thorax of *Ae. aegypti* mosquitoes. This indicates that both the midgut infection barrier and the salivary gland barrier prevent infection and subsequent transmission of SHUV by *Ae. aegypti* mosquitoes. Of the *Cx. p. pipiens* mosquitoes that were orally exposed to SHUV, none became infected during 10 days of incubation at 25°C. Moreover, replication of SHUV was low in *Cx. p. pipiens*, as evidenced by low titers when it was directly injected into the thorax. However, a relatively high percentage of mosquito saliva samples contained SHUV. We therefore conclude that the midgut barrier is the main barrier that prevents infection of *Cx. p. pipiens* with SHUV. Our findings are in line with an earlier study on the closely-related SBV, which showed no evidence for involvement of *Cx. pipiens* in virus transmission, although SBV was able to infect *Cx. pipiens* mosquitoes (Manley *et al.*, 2015). However, as *Cx. theileri* has been identified as a vector of several other bunyaviruses, this mosquito may also be a possible vector of SHUV (van Eeden, Zaayman, *et al.*, 2014; Venter, 2018). Thus, vector competence studies with additional mosquito species collected from the field are needed to fully understand the possible role of mosquitoes in natural transmission cycles of SHUV.

In this study, we determined infection, dissemination, and transmission of SHUV by infectivity assays and virus titers by EPDA (*i.e.* assays based on inoculation of samples on

Vero cells which are then screened for CPE). Such infectivity assays and EPDAs have the advantage of detecting infectious virus particles, whereas other methods like qPCR that quantify genome equivalents, may include defective virus particles and thereby not accurately represent infectious virus. Of note, observed CPE in the infectivity assays and EPDAs was found to invariably correspond with SHUV RNA as determined by RT-qPCR (S1 Supporting Information).

Recent outbreaks of SBV and BTV exemplified the tremendous impact of midge-borne viruses on animal health (Saegerman *et al.*, 2008; Beer *et al.*, 2013). Our study demonstrates highly efficient infection, replication, and dissemination of SHUV in two biting midge species (*C. nubeculosus* and *C. sonorensis*). However, conclusive evidence for SHUV transmission by biting midges should be provided by experiments with infected biting midges and susceptible mammals, although these kind of experiments are costly and complex. We cannot exclude that results obtained with laboratory-reared vectors are different from those obtained with field-collected vectors. Therefore, future studies should test vector competence of field-collected *Culicoides* biting midge and mosquito species exposed to different quantities of SHUV, to more accurately predict the risk of SHUV transmission in specific areas. These experiments in combination with behavioural and ecological research will contribute to our understanding of the transmission cycle of SHUV.

Acknowledgements

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Supplementary

S1 Supporting Information: RT-qPCR validation of Shuni virus read-out based on cytopathic effects.
doi: 10.1371/journal.pntd.0006993.s001

S1 Data: Dataset Shuni virus growth curves.
doi: 10.1371/journal.pntd.0006993.s002

S2 Data: Dataset Shuni virus vector competence experiments.
doi: 10.1371/journal.pntd.0006993.s003

Supplement 1: Validation of Shuni virus read-out based on cytopathic effects

Materials and Methods

To confirm that the observed cytopathic effects (CPE) in the infectivity and endpoint dilution assays (EPDA) was induced by Shuni virus (SHUV) a reverse transcriptase quantitative PCR (RT-qPCR) was performed on supernatants of inoculated Vero cell monolayers. A selected subset of samples including Schmallenberg virus (SBV) and SHUV stocks (positive virus controls), cell-culture medium (negative controls), non-virus exposed biting midges and mosquitoes, and SHUV-exposed biting midges and mosquitoes that tested negative or positive for SHUV infection in the infectivity assays were inoculated with a monolayer of Vero cells in a 96-well plate (Fig. S1A). After 2-3 h the inoculum was removed and replaced by 100 µl of fully supplemented HEPES-DMEM medium. Wells were subsequently scored for virus-induced CPE at 7 days post inoculation. Each well was imaged through a Dino-Eye ocular (Dino-Lite Europe, Naarden, The Netherlands) using a light microscope (Fig. S1B). The 100 µl supernatant was subsequently removed from each well and added to an Eppendorf tube containing 300 µl Trizol-LS (Thermo Fisher Scientific, Massachusetts, United States) to inactivate the virus. The 96-well plate was fixed with 4% paraformaldehyde and washed three times with phosphate buffered saline before the remaining cells were stained with 50 µl crystal violet (Sigma-Aldrich, Missouri, United States; Fig. S1C). After 10 minutes the plate was washed three times with water and air dried before imaging. The supernatant samples in Trizol-LS were used for SHUV-specific RT-qPCR (Fig. S1D).

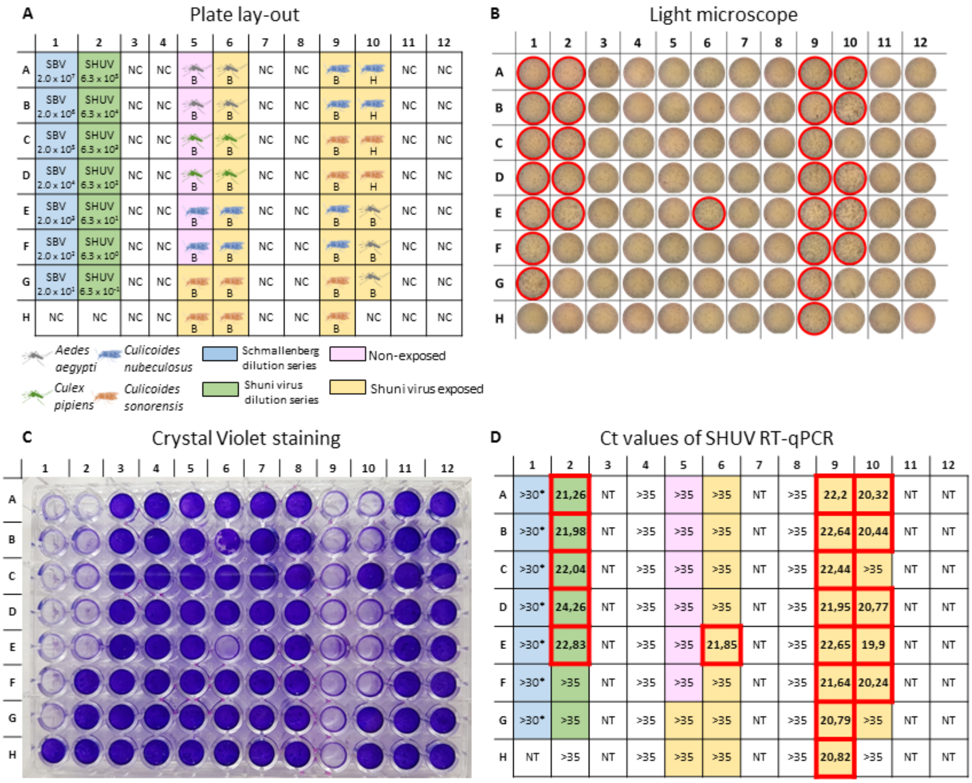
Viral RNA was isolated from the Trizol-LS samples with the Direct-Zol™ RNA Miniprep kit (Zymo Research, California, United States) according to the manufacturer's instructions. The SHUV specific RT-qPCR was designed (PrimerQuest Tool; Integrated DNA Technologies, Iowa, United States) using the S-segment sequence (Genbank: KU937313.1). Primers and probe sequences are given in table S1. The LightCycler RNA Amplification Kit HybProbe (Roche, Almere, The Netherlands) in combination with a LightCycler 480 system (Roche) were used for a one-step RT-qPCR reaction. Cycling conditions were as follows: reverse transcriptase at 45°C for 30 min, denaturation at 95°C for 5 min, 40 cycles of 5 s at 95°C and 35 s at 57°C.

Table S1. Shuni virus primers and probe for qPCR.

	Sequence
Forward primer	5'-GAAGGCCAAGATGGTACT-3'
Probe	5'-FAM-AGTAAGACGGCACAACCGAGTGTT-BHQ1-3'
Reverse primer	5'-CAATACACAGCAAATCCTGT-3'

Results and conclusions

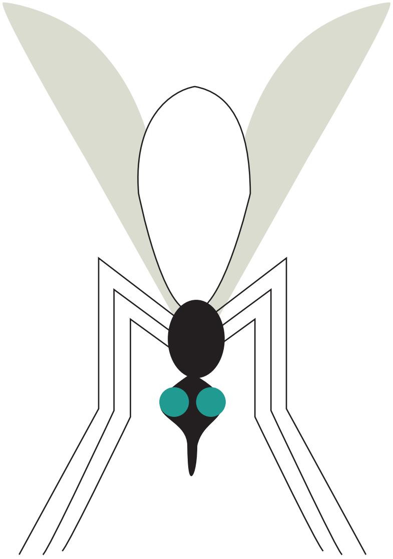
As shown in Figure S1, a strong correlation between CPE and a RT-qPCR positive result was observed. All CPE presenting wells initially inoculated with a serially diluted SHUV stock presented a high signal in the PCR. Of note no specific signal was observed with a SBV serially diluted stock. Importantly, no CPE or PCR positive signal was observed with SHUV negative bodies. A high SHUV-specific PCR signal was observed with samples showing CPE. Altogether, this experiment confirms that the infectivity assays are a reliable tool for detecting infectious SHUV in biting midge and mosquito samples.



	1	2	3	4	5	6	7	8	9	10	11	12
A	SBV 2.0×10^7	SHUV 6.3×10^5	NC	NC	B	B	NC	NC	B	H	NC	NC
B	SBV 2.0×10^6	SHUV 6.3×10^4	NC	NC	B	B	NC	NC	B	H	NC	NC
C	SBV 2.0×10^5	SHUV 6.3×10^3	NC	NC	B	B	NC	NC	B	H	NC	NC
D	SBV 2.0×10^4	SHUV 6.3×10^2	NC	NC	B	B	NC	NC	B	H	NC	NC
E	SBV 2.0×10^3	SHUV 6.3×10^1	NC	NC	B	B	NC	NC	B	B	NC	NC
F	SBV 2.0×10^2	SHUV 6.3×10^0	NC	NC	B	B	NC	NC	B	B	NC	NC
G	SBV 2.0×10^1	SHUV 6.3×10^{-1}	NC	NC	B	B	NC	NC	B	B	NC	NC
H	NC	NC	NC	NC	B	B	NC	NC	B	NC	NC	NC

	1	2	3	4	5	6	7	8	9	10	11	12
A	>30*	21,26	NT	>35	>35	>35	NT	>35	22,2	20,32	NT	NT
B	>30*	21,98	NT	>35	>35	>35	NT	>35	22,64	20,44	NT	NT
C	>30*	22,04	NT	>35	>35	>35	NT	>35	22,44	>35	NT	NT
D	>30*	24,26	NT	>35	>35	>35	NT	>35	21,95	20,77	NT	NT
E	>30*	22,83	NT	>35	>35	21,85	NT	>35	22,65	19,9	NT	NT
F	>30*	>35	NT	>35	>35	>35	NT	>35	21,64	20,24	NT	NT
G	>30*	>35	NT	>35	>35	>35	NT	>35	20,79	>35	NT	NT
H	NT	>35	NT	>35	>35	>35	NT	>35	20,82	>35	NT	NT

Figure S1. Validation of Shuni virus (SHUV) infection assay. Serial diluted Schmallenberg virus (SBV) and SHUV stocks, homogenates of non-exposed *Aedes aegypti, Culex pipiens* and *Culicoides nubeculosus*, and SHUV exposed vector species (*Ae. aegypti, Cx. pipiens, C. nubeculosus* and *C. sonorensis*) were added to a 96 wells plate with Vero cells as indicated in panel A. B = whole body, H = head, NC = negative control. Panel B: Light microscope images of cells at 7 days post inoculation. Red lining indicates presence of cytopathic effects. Panel C: Crystal violet staining of Vero cells in the 96 wells plate following the 7 days incubation. Panel D: SHUV RT-qPCR of the supernatants obtained at 7 days post inoculation. Red lining indicates samples with low Ct values. NT = not tested, * = very low signal with no sigmoidal amplification which is probably due to some very low level cross-reactivity between the SHUV primers and probe with the SBV S-genome segment.



Chapter 10

General discussion

Introduction

With a constantly growing human population, increased travel and trade, and climate change, we face new challenges related to vector-borne diseases, such as food security and increased risks of infectious diseases. Global challenges associated with vector-borne diseases need integrated solutions to improve animal and human health. These challenges should therefore be tackled with a One Health approach that includes human, veterinary, wildlife, environmental and ecological aspects to develop durable solutions and increase our preparedness for future disease outbreaks in animals and humans (Garros *et al.*, 2018).

After the eradication of malaria, Europe has remained relatively free from mosquito-borne pathogens. However, since the beginning of the new century, there have been frequent endemic outbreaks of animal and human diseases caused by vector-borne viruses. Mosquito-borne viruses such as WNV and USUV affected bird populations. Moreover, biting midge-borne viruses such as BTV and SBV caused major outbreaks in ruminants (Mellor *et al.*, 2000; Buckley *et al.*, 2003; Van Schaik *et al.*, 2008; Wernike *et al.*, 2012; Gubbins *et al.*, 2014; Saegerman *et al.*, 2014; Veldhuis *et al.*, 2014). At the same time, viruses such as CHIKV, DENV, and WNV, have resulted in hundreds of human cases and dozens of deaths on the European continent (Hubálek & Halouzka, 1999; Rezza *et al.*, 2007; Calistri *et al.*, 2010; Grandadam *et al.*, 2011; Sambri *et al.*, 2013; Delisle *et al.*, 2015; ECDC, 2018). There is an increased concern that more vector-borne viruses such as equine encephalitis, AHSV, or SHUV will make their way into Europe (Schaffner *et al.*, 2009). Being prepared for potential virus outbreaks is the first step to limit fast spread and thereby reduce the incidence of diseases in both animals and humans (Fig. 1).

Vector-borne diseases are the result of intricate interactions among pathogens, vectors, and hosts. Preparedness for future vector-borne disease outbreaks can be improved by better understanding these interactions, and through the development of effective control measures. Early warning systems for disease outbreaks can be improved by optimizing predictions of areas most at risk of disease outbreaks, based on monitoring and identification of vector species. In addition, endemic and exotic vector species should be tested for their ability to transmit emerging viruses. Finally, testing of wildlife, or sentinel herds for pathogens can help to predict potential disease outbreaks at an early stage.

To reduce the impact of vector-borne disease outbreaks, effective vector control measures are needed. Vector populations can be reduced by treatment of larval habitats, insecticide spraying against adults, release of sterile males, and deployment of adult traps (Benedict & Robinson, 2003; Homan *et al.*, 2016; Melo *et al.*, 2016; Meloni *et al.*, 2018). In addition to vector control, disease outbreaks can be contained by targeting the pathogen. Either by vaccination or drug treatment of the host, or by modification of the vector's capacity to transmit a pathogen. Reducing pathogen infection in vectors can for example be achieved by manipulation of their associated bacteria (Cirimotich, Ramirez*,* *et al.*, 2011; Iturbe-Ormaetxe *et al.*, 2011; Dennison *et al.*, 2014). Finally, reducing the movement of vectors or hosts will contain the pathogen in a restricted area, although this will be less effective for vector-borne diseases.

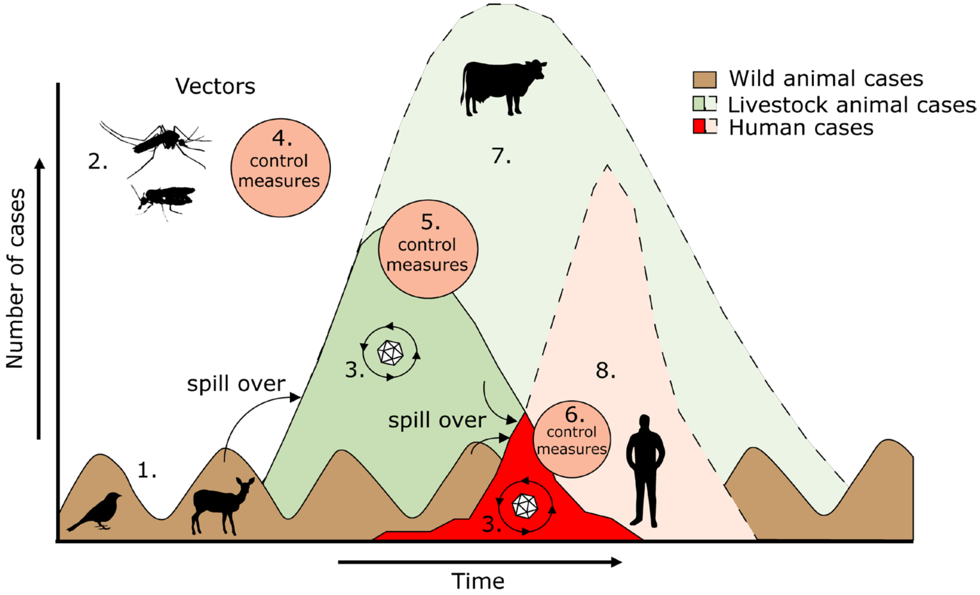


Figure 1. Disease ecology of vector-borne viruses. Natural circulation of viruses in wildlife populations (brown). A peak in disease prevalence can result in spill over of the virus to livestock (green) or humans (red). Rapid response and effective control measures can reduce further disease incidence in animals (dotted line, light green) and humans (dotted line, light red). (1) Preparedness for vector-borne disease outbreaks can be enhanced by early detection of pathogen circulation in wildlife. (2) Monitoring and identification of vector populations can improve predictions of potential hotspots for disease outbreaks. (3) Replication of the virus in animal and human populations. (4) Control measures to reduce vector populations can reduce further spread of the virus and subsequently reduce cases in animals and humans. (5) Movement restrictions in combination with vaccination or drug treatment of animals can further reduce incidences. (6) Vaccination or drug treatment of humans can reduce disease cases. (7) Benefits of reduced cases in animals. (8) Benefits of reduced cases in humans. Figure adjusted from Karesh *et al.* (2012).

This thesis aimed to acquire knowledge about European vector populations, their associated bacteria, and their ability to transmit pathogens, to increase preparedness for vector-borne infectious diseases in Europe. This was achieved by first investigating the influence of habitat type and latitude on the community composition and abundance of mosquito and biting midge species in Europe. Second, the obtained results of European vector community composition were used to predict disease risk, based on mathematical models. Third, the interactions among bacteria, viruses, and mosquitoes and biting midges were studied to find out to what extent gut bacteria of these vectors affect virus infection and transmission. In addition, I aimed to elucidate how bacterial communities change during the life cycle of biting midges, and if trans-stadial transmission of bacteria occurs. Based on this knowledge, specific bacterial species have been designated as potential candidates for paratransgenic control of vector-borne viruses. Furthermore, the data in this thesis show to what extent the gut bacterial communities of adult female biting midges are different among multiple laboratory-reared and field-collected species, and how these communities are influenced by the environment. Differences in gut bacterial communities may explain differences in infection rates among vector species. Because vector species are not equally good at transmitting pathogens, I tested a selected number of endemic and exotic mosquito and biting midge species for their ability to transmit a potential zoonotic virus that has been circulating in Africa, but has thus far not entered Europe. This general discussion will integrate the results presented in the different chapters and addresses possible implications and future perspectives.

Diversity of mosquito and biting midge communities differs among countries

Preparedness for future disease outbreaks can be enhanced by the identification of endemic and exotic vector species and monitoring of their abundance in specific regions and habitat types. Previous studies linked the spread of pathogens and subsequently diseases, to the richness and diversity of vector species in a community (Keesing *et al.*, 2006; Chaves *et al.*, 2011; Roche *et al.*, 2013). Lower vector species diversity or higher species richness were linked to an increased total vector abundance and consequently led to an increased disease risk (Keesing *et al.*, 2006; Chaves *et al.*, 2011; Roche *et al.*, 2013). I expected that areas in countries at different latitudes, as well as habitat types, would influence local vector species richness and diversity. These could subsequently indicate areas most at risk of disease spread. The results presented in chapters 2 to 5 of this thesis show that vector community composition is more defined by geographic distance between areas than by a specific habitat type. This conclusion is consistent for both mosquito and biting midge communities. Since differences in vector communities were previously linked to differences in disease risk (Keesing *et al.*, 2006; Chaves *et al.*, 2011; Roche *et al.*, 2013; Vogels *et al.*, 2017), our results suggest that differences in successful disease spread in Europe may be linked to regional differences in vector communities.

Chapters 2 and 4 of this thesis show that geographically distant vector communities are different and may indeed explain why there are regional differences in transmission. Furthermore, regional differences in disease spread were suggested to depend on factors such as husbandry practices, stocking densities, land-use, and meteorological conditions (Gubbins *et al.*, 2014). Based on results in chapters 7 and 8, I suggest that in addition to these factors, variation in midgut bacteria within species from different geographic locations might influence their vector competence. Local variation in environmental factors such as breeding habitat or microclimate temperature variation among farms, in combination with relative abundance of vector species and their associated bacterial communities, might explain the farm-to-farm differences in disease prevalence.

Previous work has identified a correlation of increased disease risk, with species richness and species diversity. However, both correlations were also based on the correlation of increased disease risk with total vector abundance (Chaves *et al.*, 2011; Roche *et al.*, 2013). Our data only showed a correlation of biting midge species diversity with total biting midge abundance, and not for biting midges species richness or mosquito species richness and diversity (chapters 2 and 4). The lowest species diversity, and highest abundance of biting midges was recorded for Italian farms. This indicates that farms in Italy are most at risk of *Culicoides*-transmitted diseases. The dynamic transmission model as presented in chapter 6 confirms that Italy had consistently high reproductive ratios, and thus a relatively high transmission risk throughout the season. On the contrary, Sweden and The Netherlands only had a single peak of high BTV reproductive ratios in July and August. This means there is only a short period of risk for sustained pathogen transmission for Sweden and The Netherlands.

Furthermore, the abundance of biting midges was highest around farms across all countries (chapter 4). The high abundance of *C. obsoletus* s.s. and *C. scoticus* around livestock farms might depend on factors related to the presence of productive breeding places such as livestock identity, stocking densities, or husbandry practices. This highlights the importance of biting midge population control at livestock farms at the appropriate time, and during disease outbreaks. However, thus far methods that target larval habitats or adult resting sites, application of repellents or insecticides on host animals, or housing livestock in screened buildings, are not yet sufficiently effective to drastically reduce biting midge populations (Carpenter *et al.*, 2008; Benelli *et al.*, 2017).

Despite differences in vector communities across latitudes, a core community of about ten species was identified for mosquitoes and biting midges. *Culex pipiens* mosquitoes and Obsoletus group biting midge species were the most abundant vectors across the study areas (chapters 2 and 4). These species are thought to play a key role in pathogen transmission in Europe, with *Cx. pipiens* as principal vector of WNV and species of the Obsoletus group as main vector of BTV and SBV in large parts of Europe. *Culex pipiens* consists of two biotypes, which have unique traits regarding their reproduction and behaviour. Similarly, species in the Obsoletus group show distinct preference for breeding habitat and attraction to hosts. Because both *Cx. pipiens* and species in the Obsoletus group are implicated as principal vectors of pathogens in Europe, it is crucial to understand the behaviour and relative distribution of these biotypes and species. Shifts in relative abundance of *Cx. pipiens* biotypes and their hybrids, or of different species of the Obsoletus group, are expected to have an effect on pathogen transmission and the reproductive ratio (Vogels *et al.*, 2017).

The *Cx. pipiens* biotype *pipiens* prefers to bite birds, whereas the *molestus* biotype prefers to bite mammals. Both biotypes can transmit WNV, but the *pipiens* biotype is expected to be most important in the enzootic cycle between birds and mosquitoes. In addition, it was predicted that higher proportions of the *pipiens* biotype increase the likelihood of WNV disease establishment (Vogels *et al.*, 2017). Based on results in this thesis, this would mean that countries at northern latitudes are more at risk of disease establishment (chapter 3). However, not only the presence of specific mosquitoes, but also temperature plays an important role in pathogen transmission. Lower temperatures are related to lower transmission rates, which decrease the risk of disease spread for northern countries (Vogels *et al.*, 2016). Ultimately, the interplay between relative abundance of competent vectors, ambient temperature, presence of the virus, and host availability will determine the risk of successful virus transmission.

The results in chapter 5 of this thesis confirm that *C. obsoletus* s.s. and *C. scoticus* (the Obsoletus complex) can be found throughout Europe at all latitudes and in all habitat types (Meiswinkel *et al.*, 2008; Nielsen *et al.*, 2010; Goffredo *et al.*, 2016; Magliano *et al.*, 2018). The widespread occurrence of *C. obsoletus* s.s. and *C. scoticus* throughout Europe, in combination with their high abundance around livestock farms, and ability to disperse over long distances (Sanders *et al.*, 2017), make them the most likely candidates for rapid farm-to-farm transmission of arboviruses in Europe. The control of biting midges will, therefore, be most efficient if it would initially focus on these two species. A better understanding about their ecology, including preferred breeding sites, host preference, biting rates, and overwintering strategies will contribute to the development of more efficient control measures against these important biting midge vectors.

Biting midge capture data predict areas at risk of BTV transmission

Preparedness for future vector-borne disease outbreaks can be improved by predictions of areas at risk of pathogen transmission by vectors. Data on the distribution and abundance of field-collected vectors can be used in mathematical models to improve these predictions. With this information, response strategies can be targeted to efficiently deploy control measures to stop further spread of diseases (Anyamba *et al.*, 2009).

Based on the data presented in this thesis, species diversity or species richness were not reliable measures to predict disease risk. To obtain a better estimation for risk of disease transmission, we linked trap catches with climate data, and seroprevalence in cattle during the BTV outbreak of 2007 (chapter 6; Santman-Berends *et al.*, 2010; Santman-Berends *et al.*, 2013). This provided a prediction of cattle and sheep herds at risk of sustained transmission. The model in chapter 6 showed that most of the naïve livestock herds in Europe were at risk of sustained transmission of BTV over the past decade. This implies that sufficient susceptible wildlife and livestock in a region, in combination with the introduction of an arbovirus transmitted by biting midges, would likely lead to a disease outbreak. Large-scale outbreaks in Europe have not occurred in the last decade, most likely because sufficient animals had immunity obtained during previous virus circulation, or from vaccination programs (Savini *et al.*, 2008). However, the birth of new susceptible animals, and introduction of a new virus or virus strain can lead to new outbreaks of vector-borne diseases. Through monitoring and identification of potential vectors in different regions and habitats, in combination with vector competence studies on local mosquitoes and biting midges, we can increase the reliability of predictions for areas most at risk of disease outbreaks. Based on these predictions, early warning systems can be deployed in areas that are likely candidates for disease spread. With the inclusion of longitudinal data on vector abundance and species occurrence throughout Europe, the current model could make substantiated predictions into the future. In addition, the model allows to make predictions into the future, based on climate prospects. Hence, we can predict how climate change will influence the risk of disease outbreaks in Europe in the coming decades.

Based on the Onderstepoort Veterinary Institute traps that I used to collect biting midges, we estimated that the expected biting rate per cow per day is around 50% of the total daily trap catch. This information is most valuable for future studies if similar trapping techniques and protocols are used. I therefore propose to develop standardized trapping methods across Europe to increase the usability of models and mosquito and biting midge capture data. Finally, the current model assumes that all known vector species are equal, whereas I know there are significant differences among species (chapters 7 and 8; Tabachnick, 1991). Differences among species in their vector competence, or abundance in specific areas and habitats, should be incorporated into the next generation of models.

Life stages and species of biting midges harbour unique bacterial communities

Current control methods cannot drastically reduce biting midge populations around livestock farms. It is therefore important to look for alternatives to control virus spread by biting midges. Studies with mosquitoes suggest that midgut bacteria can interfere with pathogen infections, and that bacteria have the potential to be used as novel control tool in the fight against vector-borne pathogens (Favia *et al.*, 2008; Mancini *et al.*, 2016; Shane *et al.*, 2018). To pinpoint bacteria that have the potential to influence virus infection in biting midges, I identified bacterial communities in different life stages and multiple species. Results presented in chapter 7 show that bacterial communities change drastically after metamorphosis of the larvae into pupae, and subsequently into adults. Therefore, I expect the contribution of bacteria acquired during the larval stage, to the bacterial community of the adult midgut to be relatively small. On the other hand, I identified some bacteria that persist throughout each life stage, which can therefore be expected to play a role in the physiology and biology of biting midges. Future studies of the involvement of these core bacteria in life-history traits of biting midges such as development time, survival, and fitness could give new insights into their intimate relation with these vector insects.

Biting midge species collected from the field, harboured unique gut bacterial communities that were mostly species specific. Species collected in a Dutch wetland habitat contained bacteria such as *Cardinium, Rickettsia,* and *Wolbachia* that are known to be associated with insect vectors (Nakamura *et al.*, 2009; Morag *et al.*, 2012; Lewis *et al.*, 2014; Mee *et al.*, 2015;

Pagès *et al.*, 2017; Pilgrim *et al.*, 2017). For mosquitoes, it was shown that these bacteria could affect the vector capacity either through effects on vector competence or manipulation of life history traits such as survival and reproduction (Zchori-Fein & Perlman, 2004; Hedges *et al.*, 2008; Iturbe-Ormaetxe *et al.*, 2011; Pilgrim *et al.*, 2017). In the current study, these bacteria were only found in biting midge species that have, so far, not been incriminated as vectors of pathogens. It could be that these bacteria reduce virus infection or replication in those biting midge species. It will be interesting to investigate if removal of these bacteria from field-collected biting midges will increase their susceptibility to viruses.

Pseudomonas bacteria were identified as core bacteria for all life stages in both laboratory-reared species, as well as for farm- and wetland-associated biting midge species (chapter 7). These opportunistic bacteria can be found in a wide range of habitats including water, soil, plants, and animals. In addition, *Pseudomonas* is known for its intrinsic resistance to antibiotics and disinfectants (Stover *et al.*, 2000)2000. This might explain why it was found in such a wide range of environments and in many of our insect samples. Furthermore, some *Pseudomonas* species are pathogens for animals and humans (Buell *et al.*, 2003)2003. However, almost all clinical cases linked to the human pathogen *Pseudomonas aeruginosa* are related to compromised host defence (Lyczak *et al.*, 2000). In contrast to these pathogenic species, *Pseudomonas* has also been shown to provide benefits to insects through the protection of eggs against harmful bacteria, detoxification of polluted larval habitats, promotion of insect growth, and facilitation of blood digestion through reduction of oxidative stress after a blood meal (Parker *et al.*, 1977; Campbell *et al.*, 2004; Peck & Walton, 2006; Wang *et al.*, 2011; Alvarez *et al.*, 2012; Senderovich & Halpern, 2013; Chavshin *et al.*, 2015; Nayduch *et al.*, 2015; Erram, 2016; Díaz-Sánchez *et al.*, 2018). Promoting the presence of these bacteria in a laboratory rearing might facilitate rearing of biting midge species that we are currently unable to maintain under laboratory conditions. It remains important to realize that the genus *Pseudomonas* consists of many species. These species have their own traits and characteristics. Identification of bacterial species and their characteristics will assist in further understanding their wide distribution and close relationship with animals, plants, and the environment (Green *et al.*, 1974; Hardalo & Edberg, 1997; Stover *et al.*, 2000).

Gut bacterial communities interfere with virus infection

Pathogen transmission depends on the interactions between the pathogen, the vector, and its host. In all of these interactions the community of associated microorganisms plays an important role (Guégan *et al.*, 2018). I aimed to elucidate if midgut bacteria play a role in arbovirus infection and transmission and, therefore, studied the interaction among bacteria, viruses, and vectors.

The data in chapter 8 show that changes in gut bacterial communities of *C. nubeculosus* significantly increased the infection with SBV. Changes in infection rate can be caused by (specific) bacteria that either facilitate or hamper virus infection. As presented in Figure 1 of chapter 1, bacteria can interfere with virus infection through activation of the vector's innate immune responses, direct competition between bacteria and viruses for resources such as lipids or vitamins, by directly blocking pathogen interaction with the vector midgut epithelial cells, or by bacterial secretion of specific anti-pathogenic molecules such as reactive oxygen or secondary metabolites (Azambuja *et al.*, 2005; Dong *et al.*, 2006; Ryu *et al.*, 2008; Xi *et al.*, 2008; Dong *et al.*, 2009; Kumar *et al.*, 2010; Cirimotich, Dong, *et al.*, 2011; Cirimotich, Ramirez, *et al.*, 2011; Joyce *et al.*, 2011; Bahia *et al.*, 2014; Jupatanakul *et al.*, 2014; Ramirez *et al.*, 2014; Hegde *et al.*, 2015; Dennison *et al.*, 2016; Barletta *et al.*, 2017; Rodgers *et al.*, 2017). To further elucidate which mechanisms influence the interaction between bacteria and virus infection in biting midges, metagenomics or metatranscriptomics can be helpful tools. These techniques generate large amounts of data on genetic information of bacteria originating from different biting midges. Transforming these large amounts of genomics data into meaningful knowledge will be an important part of these future studies. With these -omics techniques and generated data, functional genes of bacteria can be identified that are potentially involved in the interactions between bacteria, viruses, and vectors. Functional genes in untreated or antibiotic-treated biting midge samples can be compared for their presence or absence. In addition, it can provide insights into pathways that are potentially involved in these interactions. After the identification of involved genes and pathways, identification of the associated bacteria will be the logical next step.

Complementary to the genomics approach, experimental introduction of potentially involved bacterial species, such as *Asaia, Delftia* or *Sphingomonas*, in axenic and gnotobiotic biting midges and mosquitoes may provide important insights in bacterial species-specific roles in virus-vector interactions (Correa *et al.*, 2018; Guégan *et al.*, 2018; Valzania *et al.*, 2018). When bacteria that reduce virus infection can be identified, they could be used in the development of new control tools such as the introduction of these bacteria in sugar baited traps, or inoculation of larval habitats with beneficial bacterial communities (Mancini *et al.*, 2016).

Bacteria play a key role in the physiology of many insects (Crotti *et al.*, 2009; Jupatanakul *et al.*, 2014). I expect that the influence of bacteria on virus infection, as found for biting midges in chapter 8, can also be found in other virus-insect interactions. Although we did not find an effect of bacterial communities on virus infection rates in mosquitoes, previous studies with mosquitoes have shown that specific bacteria influenced virus infection and replication (Apte-Deshpande *et al.*, 2012; Apte-Deshpande *et al.*, 2014; Bourtzis *et al.*, 2014; Ramirez *et al.*, 2014). Other insect vectors, such as aphids, whiteflies, thrips, planthoppers, or leafhoppers transmit plant viruses (Whitfield *et al.*, 2015). Similar to mosquito or biting midge-borne viruses, these plant viruses are thought to interact with bacteria that are associated with the plants or the insect vectors (Gray *et al.*, 2014; Pinheiro *et al.*, 2015). If a robust system can be developed to make use of bacteria to interfere with virus infections of their vector, this could be applied to multiple virus-vector systems. At the moment, we are just starting to understand the enormous impact bacteria have on these kinds of virus-insect interactions, or of the impact of bacteria on animal and human interactions with pathogens (Lyczak *et al.*, 2000; Clemente *et al.*, 2012).

Apart from the likely role that bacterial communities play in changing infection rates, antibiotics used as treatment (chapter 8), might themselves be responsible for changed virus-vector interactions. Antibiotic use in the livestock industry results in relatively high concentrations of antibiotics in manure (Christian *et al.*, 2003; Martínez-Carballo *et al.*, 2007), and consequently in natural breeding sites of biting midges and mosquitoes. In chapter 7, we show that only few bacteria were transmitted from larvae to pupae, and eventually to adults. The effect of antibiotics in larval habitats on adult gut bacterial communities is therefore expected to be limited. However, it remains unclear where the bacterial communities of adult biting midges originate from (chapter 7). I expect that the environment does, at least partially, influence the gut microbial community of adult females. Adult female biting midges could obtain bacteria from the environment when they emerge from their larval habitat, when they feed from plants or flowers for sugar sources, or from the skin of livestock while taking a blood meal.

Antibiotic treatment of laboratory-reared species increased the diversity of gut bacterial communities of biting midges (chapter 8). Higher diversity of gut bacteria in farm-associated biting midge species compared to wetland species, therefore, hints to the possibility that these farm species were exposed to antimicrobial compounds. The use of antibiotics in the livestock sector may therefore indirectly affect susceptibility of vectors to arboviruses, which could result in higher transmission risk of SBV. To test this hypothesis, vector competence experiments with biting midges exposed to antibiotics that are commonly used in the livestock industry should be performed, using concentrations that can be found in larval breeding habitats (animal dung), as well as antibiotic concentrations on the skin, or in the blood of livestock. In addition, experiments should be performed with field-collected or laboratory-reared *C. obsoletus* s.s. or *C. scoticus* biting midges, which are the most abundant and widespread vectors in most parts of Europe (chapters 3 and 4). Both options are challenging, since survival of field-collected adult biting midges is very low, and so far, no successful rearing of Obsoletus group species has been accomplished.

Can the diversity of gut bacteria predict virus susceptibility of biting midges?

Laboratory-reared and field-collected vectors show inter- and intra-species variability in vector competence (Tabachnick, 1991). Furthermore, species biotypes or species groups occur in varying relative abundances across Europe (chapters 3 and 5). The finding that gut bacterial communities are unique for most biting midge species, suggests that the composition of gut bacterial community may explain differences in vector competence among species (chapters 7 and 8). In addition, higher diversity of gut bacteria in farm-associated Obsoletus group species compared to non-vector wetland species, hints to the possibility that gut bacterial diversity can be used as indicator for their virus susceptibility (chapter 8). I therefore explored the possibility to use the gut bacterial community to predict differences in virus susceptibility among biting midge species.

First, I correlated the diversity of gut bacterial communities of laboratory-reared *C. nubeculosus* and *C. sonorensis* with SBV virus infection rates in laboratory experiments. Based on the data presented in chapter 8, the infection rate of untreated and antibiotic-treated groups for both biting midge species were correlated with the average Shannon-Wiener diversity measures of the gut bacterial communities in the corresponding treatments. The correlation shows that increased infection rates were linked to higher bacterial diversity (Fig. 2).

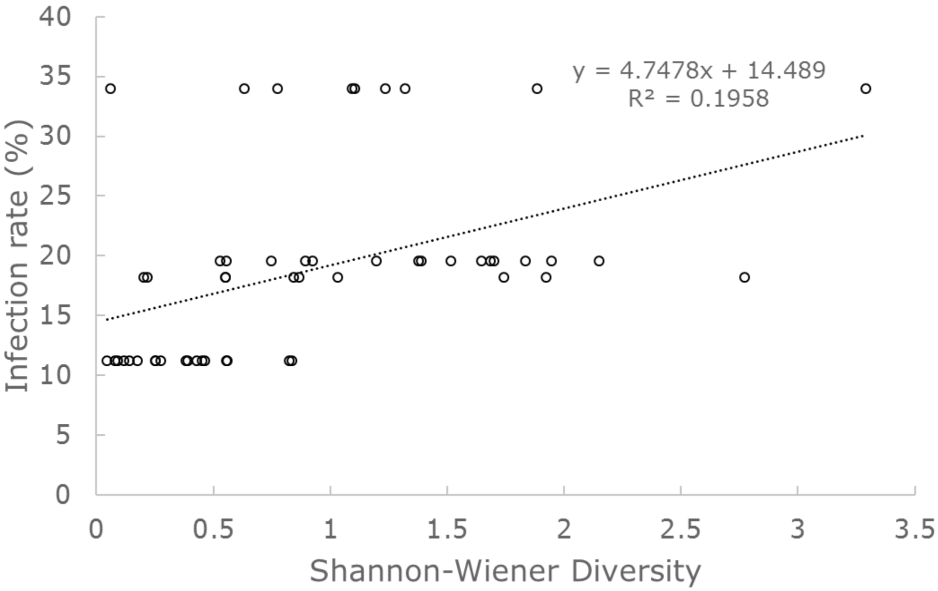


Figure 2. Correlation between Shannon-Wiener diversity of gut bacteria, and Schmallenberg virus infection rate in biting midges. The infection rates for untreated (11.2%) and antibiotic-treated (19.6%) *C. nubeculosus* and untreated (18.2%) and antibiotic-treated (34.0%) *C. sonorensis* biting midges with Schmallenberg virus. The Shannon-Wiener diversity measures were based on the gut bacterial community composition for each treatment. The correlation is represented by the dotted line ($p<0.001$).

The correlation was used to predict infection rates in nine field-collected biting midge species based on the average Shannon-Wiener diversity measures of the gut bacterial communities in the corresponding species (chapter 7; Table 1). This resulted in the fact that the five wetland-associated species have a lower expected infection rate than the farm-associated Obsoletus group species. In general, the predicted infection rates were relatively high compared to infection rates found in field-collected individuals, which were generally between 0.1% and 2.2% during SBV circulation (Balenghien *et al.*, 2014). Predicted infection rates were based on experiments performed in the laboratory, which is an artificial system and does not directly reflect what happens in a natural environment. The SBV titers we used in the laboratory experiment were 1,000 times higher than SBV titers found in infected cattle in The Netherlands during the outbreak of 2011 (Loeffen *et al.*, 2012). To obtain a more reliable estimate of infection rates in the field, estimates could be scaled based on the difference in virus titers in the blood, as well as for biting midge infection rates in the field. Infection rates found in our laboratory experiment with SBV and *C. sonorensis* were compared to a study that found BTV in *C. sonorensis* in the USA. That study found infection rates around 2%, which is about 10 times lower than the infection rates of *C. sonorensis* with SBV in our laboratory (chapter 8; Gerry *et al.*, 2001). Schmallenberg virus infection rates of field-collected biting midges of the Obsoletus complex (*C. obsoletus* s.s. and *C. scoticus*), were about 100 times lower than our estimated infection rates (Balenghien *et al.*, 2014; Elbers *et al.*, 2015). This large variation in expected infection rates will drastically change the outcome of any mathematical model. However, the relative infection rates between species will remain the same. Thus, based on these data, models could be developed to predict the effect of gut bacterial communities and species relative abundance on the risk of disease transmission.

I found a positive correlation ($R^2 = 0.20$; $p<0.001$) between diversity of gut bacterial communities and the infection rate of biting midges in the laboratory. This, in combination with the high predicted infection rates for known vectors species in the Obsoletus group, compared to lower predicted infection rates in refractory vector species such as *C. alazanicus, C. festivipennis, C. kibunensis,* and *C. pictipennis*, suggests that determination of gut bacterial diversity might be a good method to predict the susceptibility to SBV virus infection of field-collected species. The gut bacterial diversity of *C. punctatus* was the lowest of all species. Interestingly, *C. punctatus* was indicated as potential vector of BTV serotype 8 (Hoffmann *et al.*, 2009). Although its status as vector is not confirmed, this indicates that the correlation between gut bacterial diversity and virus susceptibility might be different for each virus type.

I suggest to use the current predictions of virus susceptibility for each species, to predict how differences in gut bacterial communities can influence risk of disease transmission. Predictions of virus susceptibility, in combination with presented data on relative abundance of vector species could be used as additional parameters in disease risk transmission models.

Differences in the diversity of gut bacterial communities could at least partly explain differences in infection rate. However, in our experiments only part of the individual midges became infected with SBV. The relatively large variation in the diversity of gut bacterial communities among the samples (Fig. 2), might explain why only part of the individuals became infected after ingestion of an infectious blood meal. Identification of gut bacterial communities in individuals that became infected, or did not become infected with a virus would give further insight into these individual differences.

Table 1. Estimated infection rates of laboratory-reared and field-collected biting midge species. *Culicoides nubeculosus* and *C. sonorensis* laboratory-reared biting midges were either untreated or antibiotic-treated (chapter 8). Infection rates were estimated for five biting midge species collected from wetlands in The Netherlands (NL), as well as farm-associated species from Sweden (SW), The Netherlands (NL) and Italy (IT). Estimated infection rates were calculated based on the correlation as presented in Fig. 2 ($y = 4.7478x + 14.489$), and the average Shannon-Wiener diversity measures of gut bacterial community composition for each treatment or species (chapters 7 and 8).

Species	Treatment	N	Average Shannon-Wiener diversity	Estimated infection rate (%)
C. nubeculosus	Untreated	18	0.351	4.8
C. nubeculosus	Antibiotic-treated	15	1.339	28.8
C. sonorensis	Untreated	10	1.070	22.3
C. sonorensis	Antibiotic-treated	9	1.265	27.0
C. alazanicus	NL wetland	6	1.078	22.5
C. festivipennis	NL wetland	6	0.850	16.9
C. kibunensis	NL wetland	7	0.650	12.1
C. pictipennis	NL wetland	7	0.641	11.9
C. punctatus	NL wetland	6	0.522	9.0
C. chiopterus	NL farm	3	2.144	48.4
C. dewulfi	NL farm	12	2.253	51.0
C. obsoletus s.s.	NL farm	11	1.972	44.2
C. scoticus	NL farm	11	1.216	25.8
C. dewulfi	SW farm	1	1.545	33.8
C. obsoletus s.s.	SW farm	5	1.534	33.5
C. scoticus	SW farm	14	1.184	25.0
C. obsoletus s.s.	IT farm	9	1.507	32.9
C. scoticus	IT farm	9	2.382	54.1

The mechanism that explains the influence of the gut bacterial community on virus infection rate is not yet known. It could be that changes in a specific group of bacteria, or in the abundance of gut bacteria affects the virus infection rate. The presence of specific bacteria might better explain the differences in infection rates than a general bacterial diversity measure, although bacteria such as *Asaia* and *Delftia* were hardly found in field-collected species. Bacteria commonly found in field-collected biting midges, such as *Pseudomonas, Bacillus,* Enterobacteriaceae, and *Sphingomonas* could also play a role in virus infection of biting midges. Identification of gut bacterial diversity in a larger variety of field-collected biting midge species, in combination with testing their vector competence, will increase the power of this hypothesis.

Future perspectives

The global climate will continue to change during the coming decades. Temperature is expected to increase with 0.5 °C to 2.0 °C towards 2050 (Pachauri *et al.*, 2014). Increasing temperatures lead to increased vector abundance and expansion of exotic vector species distribution range (Elbers *et al.*, 2015). In addition, the extrinsic incubation period will be reduced as viruses replicate faster at higher temperatures (McMichael *et al.*, 2006; Tabachnick, 2010; Medlock & Leach, 2015). Moreover, the human population will continue to grow, with an expected world population over 9 billion by 2050 (Cohen, 2003). The increasing demand for protein-rich food will lead to more intensified and large-scale livestock farms around the world (Delgado *et al.*, 2001; Godfray *et al.*, 2010). These congregations of animals and humans in combination with increased vector populations and faster virus replication will certainly lead to outbreaks of (new) vector-borne diseases. Results presented in this thesis show that most farms in Europe have been at continued risk of sustained BTV transmission in the past decade. The question is therefore not if, but when an outbreak will occur. The challenge will then be to be prepared for when an outbreak occurs.

Strategies to prevent vector-borne disease outbreaks are limited (Gubler, 1998; WHO, 2004), and efforts to reduce the impact of vector-borne diseases should therefore focus on preparedness and quick response strategies. For this purpose, detailed knowledge about viruses and their vectors is essential. The first step is to confirm which (endemic) vector(s) are able to transmit emerging virus strains that have the potential to cause outbreaks, such as we did for SHUV in chapter 9 of this thesis. Next, information about the vector's distribution and their abundance in combination with predictive models can indicate areas at risk of disease outbreaks (chapter 6; Purse *et al.*, 2005; Anyamba *et al.*, 2009). To facilitate comparisons and to be able to better predict how vector-borne pathogens will spread at a larger scale, I propose to use standardized protocols for monitoring of vectors across Europe. Furthermore, the identification of species biotypes and species groups should become standard in monitoring and surveillance studies, especially for known vectors such as *Cx. pipiens* and species of the Obsoletus group. This will contribute to a better understanding of their ecology and to the development of new strategies to effectively control these arthropod vectors. Targeted vector control methods such as larval control, effective adult trapping systems and the deployment of sterile males to reduce vector populations around outbreak areas should be further developed (Benedict & Robinson, 2003; Pates & Curtis, 2005; Carpenter *et al.*, 2008; Benelli, 2015; Homan *et al.*, 2016).

In addition to the control of vector populations, there are alternative strategies to prevent and control pathogen transmission. First, knowledge about the structure and pathogenicity of the virus could aid in the development of vaccines (Savini *et al.*, 2008; Kortekaas, 2014). Vaccines can be deployed in areas that are indicated by models as most at risk of disease outbreaks (Anyamba *et al.*, 2009). Second, new control methods can be developed based on bacteria associated with insect vectors. Either through enhancement of bacteria that reduce virus infection in the gut of vectors (Cirimotich, Ramirez*, et al.*, 2011), or by manipulation of stably associated bacteria, that are transstadially transmitted and found in many vector species, to use them as paratransgenic control tool against vector-borne pathogens (Favia *et al.*, 2008; Shane *et al.*, 2018).

This toolbox of monitoring and surveillance, predictive modelling, effective control strategies of vector populations, in combination with methods to reduce virus infection in vectors and mammals, will facilitate effective containment and control of disease outbreaks (Fig. 1). If a rapid response strategy is in place, it should not matter when or where a disease outbreak occurs, because we will be ready to take appropriate action.

Concluding remarks

The results presented in this thesis may improve the preparedness for potential disease outbreaks. First, by providing a mathematical model, with data on vector occurrence and abundance, to predict areas at risk of disease outbreaks. The risk of sustained transmission was relatively high throughout Europe for the past decade, and the emergence of (new) vector-borne diseases is therefore inevitable. New control tools based on bacteria that influence virus infection in biting midges, might help to reduce pathogen spread in the future. Metagenomics can be used to elucidate pathways that determine the interaction between bacteria and biting midge-borne viruses. Since most biting midge species have their own unique gut bacterial communities, we may use closely associated (core) bacteria that occur in many biting midge species, to incorporate into new control strategies. In the future, such new control tools could for example be used to reduce the transmission of Shuni virus, which was found to be transmitted by biting midges, but not by mosquitoes.

We cannot prevent the outbreaks of vector-borne diseases. However, we can be prepared to take appropriate measures when necessary. This will help to reduce the impact of disease outbreaks on animal and human health. Preparedness is largely dependent on detailed knowledge about the pathogen, its vector and hosts, as well as their interactions. Integration of knowledge from different research fields is crucial to prevent large disease outbreaks in the near future. Only with a One Health approach that includes participation of scientists (e.g. entomologists, virologists, microbiologists), and stakeholders from different integrative levels (e.g. farmers, veterinarians, public health authorities, politicians, industrial partners), can we quickly respond and reduce the impact of emerging vector-borne diseases in the coming era.

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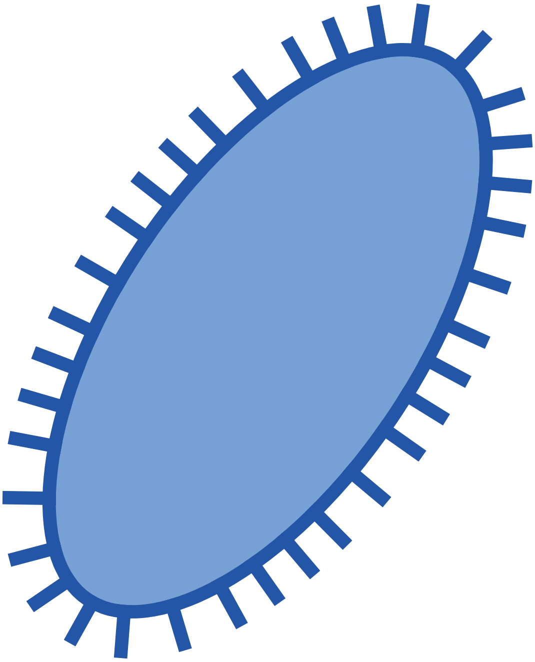

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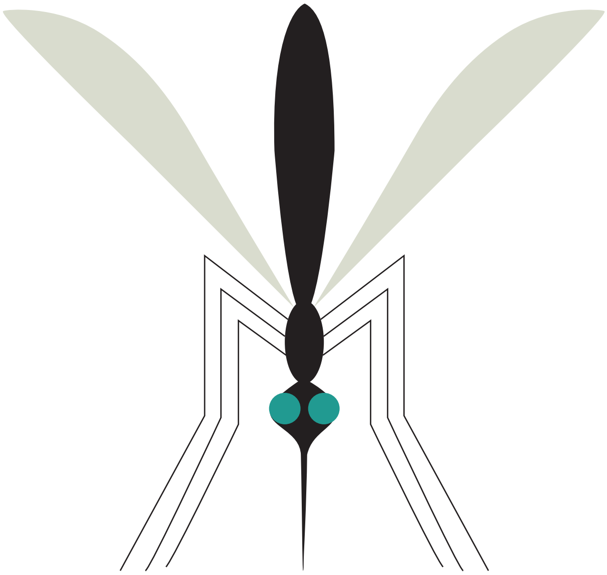
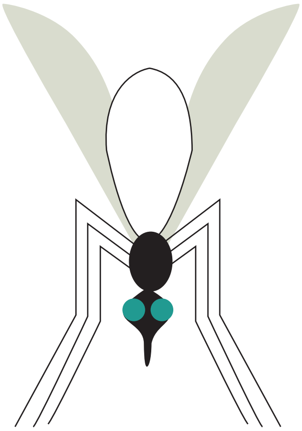




Summary

With a constantly growing human population, increased travel and trade around the globe, and global climate change, we face new challenges such as food security and increased risks of infectious diseases. Insect vectors, such as mosquitoes and biting midges, can transmit pathogens. These pathogens cause diseases in humans and animals, thereby negatively affecting health, welfare, and the economy. To tackle these challenges, solutions are needed that include human, veterinary, wildlife, environmental and ecological aspects. Therefore, the aim of this thesis was to answer the question whether environmental and geographical aspects influence vector abundance and community composition, of both the vectors and their bacterial community. With this knowledge about vector communities, I then aimed to elucidate if these field collections of biting midges can be used to predict risks of vector-borne veterinary diseases. Differences among the bacterial communities of field-collected vector species might explain differences in their vector competence. Therefore, I aimed to know whether gut bacteria can influence virus infection and transmission in mosquitoes and biting midges. Since not all vector species can transmit each virus, I finally aimed to know whether endemic and exotic mosquito and biting midge species are competent vectors of the potentially zoonotic Shuni virus.

Studies on species diversity of mosquitoes and biting midges often focus on a specific habitat, region or country, making it difficult to compare and validate results across Europe. To facilitate wider comparisons, this thesis focused on monitoring species diversity of mosquitoes and biting midges in three habitat types located in three countries across Europe (chapters 2 and 4). A total of 27 locations in Sweden, The Netherlands and Italy, comprising farm, peri-urban and wetland habitats were sampled monthly from July 2014 to June 2015, except for the winter months. Indices of species richness, evenness, and diversity were calculated. In addition, vector community compositions were analysed using non-metric multidimensional scaling.

A total of 11,745 female mosquitoes and 50,085 female biting midges were trapped during 887 and 442 collection nights respectively. For both vectors, differences in species communities were more distinct across the three countries than the three habitat types. The highest diversity for mosquitoes and biting midges was found in Sweden. Comparing the habitats, species diversity was the highest at farms for mosquitoes, whereas it was lowest at farms for biting midges. Most individuals were trapped in Italy for both insect groups. A core mosquito and biting midge community could be identified for the three countries, with *Culex pipiens* and Obsoletus group species as the most abundant mosquito and biting midge species respectively. Biotypes of the *Cx. pipiens* complex and species of the Obsoletus group show differences in ecological and behavioural characteristics, that are relevant for pathogen transmission. Differences in vector communities across countries imply different patterns of disease emergence and spread throughout Europe. How specific species and their associated communities affect disease risk remains unclear. However, *Cx. pipiens* and species in the Obsoletus group are expected to have a substantial contribution to the spread of vector-borne pathogens in Europe.

Culex pipiens is the main vector of West Nile virus in Europe. This mosquito species consists of two morphologically identical biotypes, *pipiens* and *molestus*, which can form hybrids.

Until now, population studies of *Cx. pipiens* had not differentiated between biotypes and hybrids at the European scale, nor have they used comparative surveillance approaches. I therefore aimed to elucidate the relative abundance of *Cx. pipiens* biotypes and hybrids in the three habitat types at different countries across Europe (chapter 3). Collected *Cx. pipiens* mosquitoes were identified to biotype with qPCR. From northern to southern latitudes there was a significant decrease in biotype *pipiens* and an increase in biotype *molestus*. Our results emphasize the need to differentiate *Cx. pipiens* to the biotype level, especially for proper future WNV risk assessments for Europe.

Culicoides species from the Obsoletus group are important vectors of bluetongue and Schmallenberg virus in Europe. This group consists of several species that cannot easily be identified using morphological characteristics. I aimed to elucidate the species composition of the Obsoletus group in three habitat types in different countries across Europe (chapter 5). Biting midges were identified using PCR and gel electrophoresis. Species composition was unique for most country-habitat combinations. *Culicoides chiopterus* and *C. dewulfi* were only found in substantial numbers in sample locations from The Netherlands, whereas the majority of the identified biting midges were either *C. obsoletus* s.s. or *C. scoticus*. The wide distribution of these two species across all habitat types and countries, in addition to their high abundance at livestock farms, make *C. obsoletus* s.s. and *C. scoticus* the most likely candidates for rapid farm-to-farm pathogen transmission throughout Europe. To gain more insight in the potential role of these vectors in the spread of pathogens, field data should be incorporated into mathematical models, to better assess the risk of vector-borne disease outbreaks.

Bluetongue virus (BTV) is transmitted by biting midges and has been circulating in Europe since a major outbreak occurred in 2006 and 2007. The unpredictability of the biting activity of biting midges leads to difficulty in computing accurate transmission models. In chapter 6, this thesis uniquely integrates field collections of biting midges with a multi-scale modelling approach. We inferred the environmental factors that influence the dynamics of biting midge catching, and then directly linked predicted biting midge catches to BTV transmission dynamics. Catch predictions were subsequently linked to the observed BTV prevalence amongst sentinel cattle during the 2007 outbreak in The Netherlands. With this, we were able to directly infer the bias between daily midge catch predictions and the true biting rate per cow per day. The expected biting rate per cow per day at a specific location was around 50% of the total biting midges collected with a trap in one day. Extending the model across Europe, for different seasons and years, indicated that whilst intensity of transmission is expected to vary widely from herd to herd, around 95% of naïve herds in western Europe have been at risk of sustained transmission over the last 15 years. Successful transmission however, is not only dependent on extrinsic factors that influence vectorial capacity (e.g. temperature, vector abundance, and host abundance), but also on intrinsic factors (e.g. host preference, vector physiology, infection, dissemination, and transmission success), that influence vector competence of specific mosquito or biting midge species or individuals. Recently, insect gut bacteria have been hypothesized to influence the interaction of a virus and its vector, thereby adding another factor that can influence vector competence.

Bacteria are part of the insect gut system and influence many physiological traits of their host, such as nutrient availability, development time, longevity, and reproduction. In addition, gut bacteria may even reduce or block the transmission of viruses in several species of arthropod vectors. Only a limited number of studies have investigated the bacterial communities in *Culicoides* biting midges. Understanding how bacterial communities vary among different species of biting midges, and their related life stages, will help to understand how bacterial communities can be manipulated and ultimately be used as novel tool to reduce pathogen transmission. In chapter 7, I investigated how bacterial communities are influenced by life stage, species identity, and geographic distance. To this end, the bacterial communities in multiple field-collected species from different habitats and countries, and different life stages of two lab-reared biting midges, were identified using Illumina sequencing of 16S rRNA. During the transition from the larval to the pupal stage the bacterial community drastically changed, and only *Pseudomonas*, Burkholderiaceae and *Leucobacter* bacteria were found throughout the entire biting midge life cycle. Bacterial communities among field-collected biting midges were unique for almost each species, meaning that bacterial communities of individuals within a species were highly similar, but communities among species were divergent. Besides this species identity effect, also geographic distance influenced the gut bacterial communities of farm-associated biting midges. These differences in bacterial communities among species and geography might contribute to the observed inter- and intra-species variability in vector competence, whereas stably associated bacteria such as *Pseudomonas* can be potential new candidates for paratransgenic strategies to control vector-borne pathogens.

To show how communities of gut bacteria may contribute to variability in vector competence within and among species, we investigated if gut bacteria can influence the ability of vectors to transmit arboviruses. In chapter 8, I therefore investigated the impact of gut bacteria on the susceptibility of *C. nubeculosus* and *C. sonorensis* biting midges for Schmallenberg virus, and of *Ae. aegypti* mosquitoes for Zika and chikungunya virus. Gut bacterial communities were modified by treating the adult insects with antibiotics. The gut bacterial communities were identified, and mosquito and biting midge susceptibility to arbovirus infection was tested by feeding insects with an infectious blood meal. Antibiotic treatment led to changes in gut bacterial communities, and this significantly increased infection rates of *C. nubeculosus* with Schmallenberg virus. Infection rates of *Ae. aegypti* mosquitoes with ZIKV or CHIKV did not change after antibiotic treatment. *Asaia* bacteria were abundant in untreated, and largely absent in *C. nubeculosus* biting midges and *Ae. aegypti* mosquitoes. Antibiotic treatment resulted in relatively more *Delftia* bacteria in both biting midge species, but not in mosquitoes. I conclude that the effect of gut bacteria on arbovirus infection is specific for each vector, virus, and bacterial species combination.

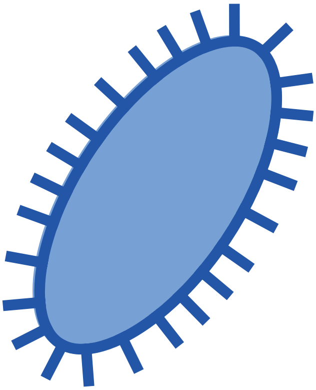
Arboviruses are notorious for causing unpredictable and large-scale epidemics and epizootics. Shuni virus (SHUV) has zoonotic potential and was recently associated with severe disease in livestock and wildlife. Although most viruses are transmitted by either mosquitoes or biting midges, isolations of SHUV from both vector species from the field suggested that SHUV may be transmitted by both. In chapter 9, I therefore tested whether laboratory-reared biting midge species (*C. nubeculosus* and *C. sonorensis*) and mosquito species (*Cx. p. pipiens* and *Ae. aegypti*), could transmit the virus. I found that SHUV was able to successfully disseminate in both biting midge species, whereas no evidence of infection or transmission in either mosquito species was found. Our results show that SHUV infects and disseminates in two different *Culicoides* species, suggesting that these insects could play an important role in the disease transmission cycle.

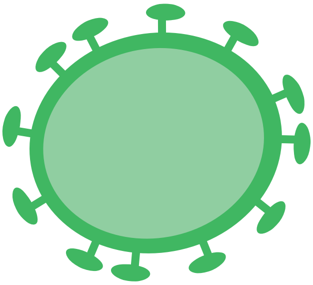
In the final chapter of this thesis, the contribution of this research to the preparedness for vector-borne infectious diseases is addressed. First, the importance of vector diversity and abundance to the contribution of disease spread is discussed. The discussion continues with the role of core species and the importance of biotypes and species groups. Furthermore, the discussion elaborates on the tripartite interaction of gut bacteria, viruses, and vectors, and how this system could be translated to other virus-insect interactions. In addition, I discuss how factors like vector community composition and their associated bacteria could influence disease risk dynamics.

I propose to use standardized protocols for monitoring of vectors across Europe, to facilitate comparisons of vector communities and to be able to better predict how vector-borne pathogens will spread at a European scale. In addition, I advise that identification of species biotypes and species groups becomes standard in monitoring and surveillance studies, especially for known vectors such as *Cx. pipiens* and biting midge species of the Obsoletus group. This will contribute to a better understanding of their ecology and to the development of new strategies to effectively control these insect vectors. I suggest that besides the control of vector populations, there may be alternative strategies to control pathogen transmission. First, knowledge about the structure and pathogenicity of the virus could aid in the development of vaccines. Second, new control methods can be developed based on bacteria that are associated with insect vectors. Either through enhancement of bacteria in the gut of vectors that reduce virus infection rates, or by manipulating stably associated bacteria, to use them as paratransgenic control tool against vector-borne pathogens.

I conclude that the results presented in this thesis improve the preparedness for potential disease outbreaks. First, by identifying mosquito and biting midge community compositions at different locations in Europe, that were used for model based predictions. Second, by providing a mathematical model that can predict areas at risk of disease transmission based on environmental factors, vector abundance, and seroprevalence data. Based on the model predictions, I conclude that the emergence of (new) vector-borne diseases is inevitable. Testing endemic and exotic vector species for their ability to transmit emerging pathogens like Shuni virus, increases the preparedness for such potential new disease outbreaks. In addition, we may use closely associated (core) bacteria that occur in many biting midge species, to incorporate into new control tools to reduce virus transmission.

Viruses will continue to cause outbreaks of diseases in animals and humans throughout the world. The integration of knowledge from different research fields will be crucial to prevent large disease outbreaks in the near future. Ultimately, with continued monitoring of vector populations in combination with predictive models, we will be able to predict when and where disease outbreaks are most likely to occur. This, in combination with tools to effectively control vector populations, and methods to reduce virus infection in hosts and vectors, will facilitate effective containment and control of future disease outbreaks.





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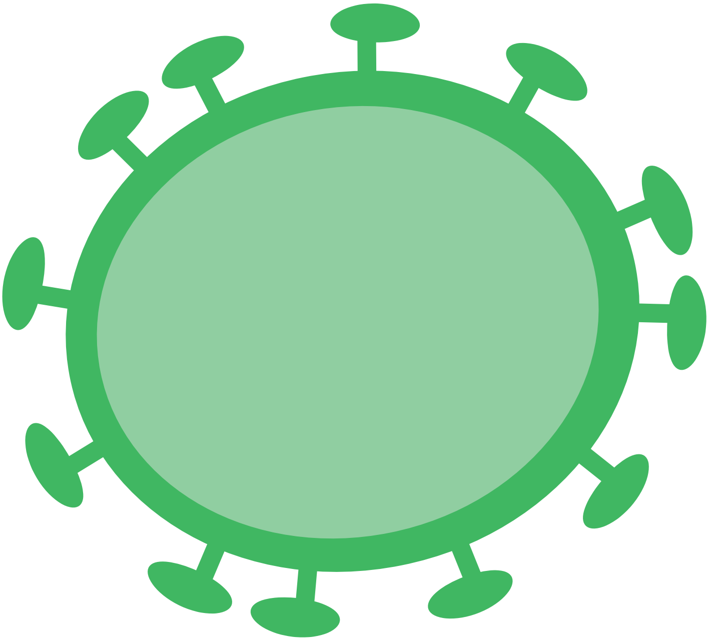
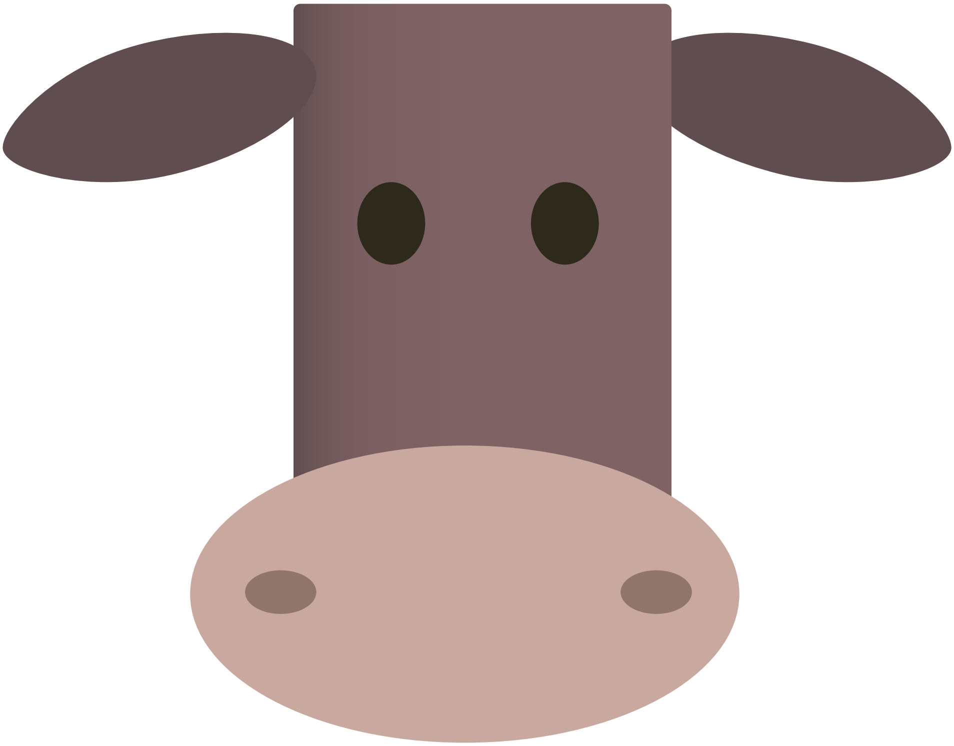


Curriculum vitae

Tim Wim Ruud Möhlmann was born on the 19th of November 1986 in Eindhoven, The Netherlands. Before he was able to walk, Tim already showed a fascination for insects. Biology was one of his favourite subjects at high school, Van Maerlant Lyceum in Eindhoven. It was not surprising that he started studying Biology in Wageningen in 2005. For his BSc thesis he studied the effect of climate change on insects. In addition to his fascination for biology and insects, he is a competitive sportsman. He served on the board of the sports association SWU Thymos for one year, before enrolling in the MSc Biology programme in Wageningen. During his study he travelled to Malawi to research the potential of starting small-scale insect farms in this country. For his MSc thesis he investigated the evolution of asexual and sexual reproduction in fungi at the Laboratory of Genetics. Thereafter, he spent six months in Benin, West-Africa, to study how weaver ants can be used as a biological control agent against fruit flies in mango orchards. After graduation he worked as manager and researcher for an international insect rearing company. In 2014 he started as a research associate at Wageningen University in a European project investigating mosquito and biting midge communities. He continued with this work at Linköping University in Sweden, which resulted in the first three experimental chapters of this thesis. Simultaneously, he started his own business in selling ants as pets and giving lectures about the fascinating world of ants. This gave him the opportunity to share his passion for keeping insects with a wider public. His career continued with a position as junior researcher at Plant Research International, in the Bio-interactions group at Wageningen University and Research. This set the basis for the work presented in the final chapters of this thesis, on the interaction among bacteria, virus particles, and vectors. At the Laboratory of Entomology he continued this work in a PhD position. Finally, the combination of previous work resulted in this elaborate PhD thesis. Tim will continue with his ant business, in combination with a job at a company that provides innovative mosquito traps around the world.



List of publications

Published articles in a journal

Möhlmann T.W.R., Wennergren U., Tälle M., Favia G., Damiani C., Bracchetti L., Koenraadt C.J.M. (2017) Community analysis of the abundance and diversity of mosquito species (Diptera: Culicidae) in three European countries at different latitudes. Parasites & vectors. 10(1):510. (chapter 2 in this thesis)

Vogels C.B.F.*, **Möhlmann T.W.R.***, Melsen D., Favia G., Wennergren U., Koenraadt C.J.M. (2016) Latitudinal diversity of *Culex pipiens* biotypes and hybrids in farm, peri-urban, and wetland habitats in Europe. PLoS One. 11(11):e0166959. (chapter 3 in this thesis)

Möhlmann T.W.R., Wennergren U., Tälle M., Favia G., Damiani C., Bracchetti L., Takken W., Koenraadt C.J.M. (2018) Community analysis of the abundance and diversity of biting midge species (Diptera: Ceratopogonidae) in three European countries at different latitudes. Parasites & Vectors. 11(1):217 (chapter 4 in this thesis)

Möhlmann T.W.R., Oymans J., Wichgers Schreur P.J., Koenraadt C.J.M., Vogels, C.B.F. (2018) Vector competence of biting midges and mosquitoes for Shuni virus. PLoS Neglected and Tropical Diseases. 12(12): e0006993 (chapter 9 in this thesis)

Submitted manuscripts to a journal

Möhlmann, T.W.R., Keeling, M.J., Wennergren, U., Favia, G., Santman-Berends, I., Takken, W., Koenraadt, C.J.M., Brand, S.P.C. Biting midge dynamics and bluetongue transmission: A multiscale model linking catch data with climate and disease outbreaks. Submitted. (chapter 6 in this thesis)

Möhlmann, T.W.R., Bekendam, A.M., van Kemenade, I., Wennergren, U., Favia, G., Takken, W., Koenraadt, C.J.M. Latitudinal diversity of biting midge species within the Obsoletus group across three habitats in Europe. Submitted. (chapter 5 in this thesis)

Koenraadt, C.J.M., **Möhlmann, T.W.R.,** Verhulst, N.O., Spitzen, J., Vogels, C.B.F. Effect of overwintering on survival and vector competence of the West Nile virus vector *Culex pipiens*. Submitted.

Other publications

Koenraadt, C.J.M., **Möhlmann, T.W.R.**, Vogels, C.B.F., Steen, W. Bunkers nieuwe Hollandse waterlinie vol met steekmuggen. Nature Today: 13-03-2015

Möhlmann, T.W.R. Mens en mier, een intieme relatie. Dierplagen: Jaargang 21. Nr. 2. 2018

Möhlmann, T.W.R., Koenraadt, C.J.M. Nieuwe steekmug in Nederland ontdekt. Nature Today: 14-11-2016

Training and education statement

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

The C.T. De Wit
Graduate School
PE&RC
PRODUCTION
ECOLOGY
& RESOURCE
CONSERVATION

Review of literature (4.5 ECTS)

- The effect of communities in pathogen transmission by mosquitoes and biting midges

Writing of project proposal (1.2 ECTS)

- The role of mosquito and biting midge diversity and their microbial community on the spread of pathogens

Post-graduate courses (4.2 ECTS)

- Theoretical Biology course; Linköping University, Sweden (2016)

Laboratory training and working visits (1.5 ECTS)

- BSL3 training course; RIVM (2017)

Invited review of (unpublished) journal manuscript (2 ECTS)

- Parasites & Vectors: *Culicoides* distribution and abundance (2018)
- Parasites & Vectors : *Culicoides* Obsoletus group spp. distribution (2018)

Deficiency, refresh, brush-up courses (3.1 ECTS)

- Fundamental and Applied Virology; WageningenUR (2017)
- Preventing the Zika Virus: understanding and controlling the Aedes Mosquito; London School of Hygiene & Tropical Medicine, online (2016)

Competence strengthening / skills courses (5 ECTS)

- Scientific artwork with Adobe Photoshop and Illustrator; WageningenUR (2016)
- Adobe InDesign essential training; WageningenUR (2016)
- Wageningen career day; WageningenUR (2017)
- Research integrity; WageningenUR (2017)
- Communication with the media and the general public; WageningenUR (2017)
- Reviewing a scientific paper; WageningenUR (2017)
- Scientific writing; WageningenUR (2018)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.5 ECTS)

- Wageningen PhD symposium: diversity of science (2016)
- PE&RC Last year weekend (2017)
- WGS PhD Workshop carousel (2018)
- Wageningen PhD symposium: bridging science & society unifying knowledge (2018)

Discussion groups / local seminars / other scientific meetings (5.7 ECTS)

- Nederlandse entomologische vereniging annual meeting (2014-2016)
- PhD Lunch meetings (2014-2017)
- Wageningen evolution and ecology seminars (2014-2017)
- European society of vector ecology workshop on *Culicoides* identification and ecology (2016)
- Dutch Arboviral Network meeting (2016, 2018)
- The Netherlands Centre for One Health masterclass (2017)
- R Sessions on microbiota data (2017-2018)
- Symposium on Global One Health; Wageningen University (2018)
- Netherlands Centre for One Health annual scientific meeting (2018)

International symposia, workshops and conferences (10.3 ECTS)

- Livestock epidemics (LIVEepi) meetings (2014-2016)
- European society of vector ecology conference (2016)
- International society of vector ecology conference (2017)
- European society of vector ecology conference (2018)

Lecturing / Supervision of practicals / tutorials (7.1 ECTS)

- Basics of infectious diseases, practical (2016, 2017)
- Frontiers of infectious diseases, practical (2017)
- Frontiers in medical and veterinary biology (2017)
- Supervision of BSc student (2017)

Supervision of MSc students (3 ECTS)

- Dutch biting midge habitat and microbiome identification on farms

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Thesis lay-out: Tim Möhlmann

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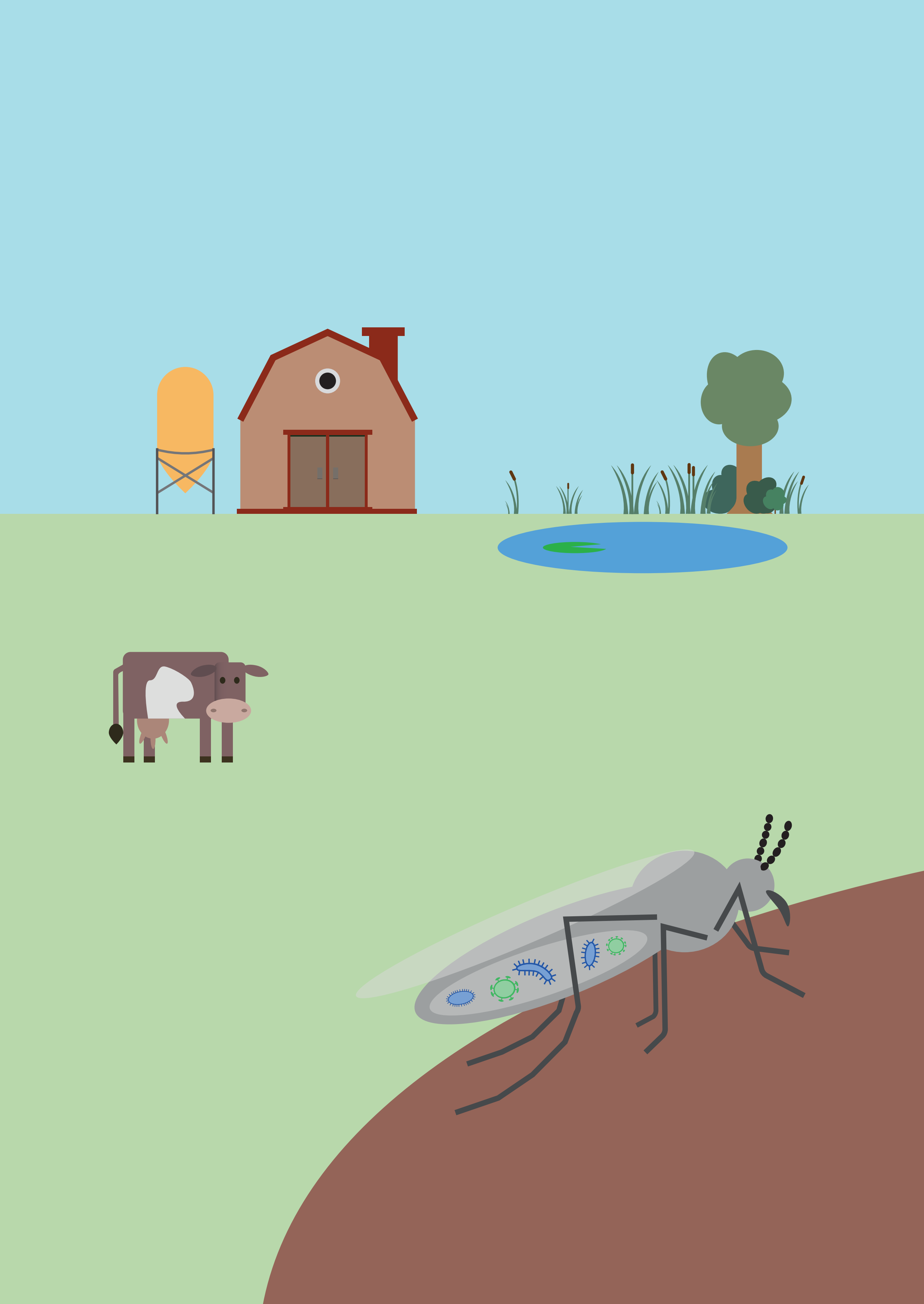
Propositions

1. Midgut bacteria are a key factor in virus-vector interactions.
   (this thesis)
2. Biting midges are the most important vectors of Shuni virus.
   (this thesis)
3. Infectious disease outbreaks in wildlife are caused by humans rather than by nature.
4. Articles with the highest impact are published without a peer review process.
5. Being lazy increases productivity.
6. The selection of insect species that are mass-reared for feed and food was as improvident as the selection of our most used crops and farm animals.
7. Happiness comes from the work environment, not from work itself.
8. Ants are the best pets one can have.

Propositions belonging to the thesis entitled

‘Mosquitoes, midges and microbiota
European vector diversity and the spread of pathogens’

Tim W. R. Möhlmann
Wageningen, 22 March 2019