Biting midges (Diptera: Ceratopogonidae) of the genus *Culicoides* LATREILLE – evaluation of their role as Schmallenberg virus vectors and investigation of their ecological aspects in Germany

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1. Introduction

1.1 Biting midges and their impact as arbovirus vectors – a short historical overview

Biting midges (Diptera: Ceratopogonidae) are among the least understood and investigated dipteran families. For many years the scientific research neglected these nematoceran insects, which was mainly due to their small size. Even though ceratopogonids had been known to be able to transmit several pathogens including parasitic protozoans, nematodes, bacteria and viruses long ago, the importance of their role as arthropod-borne virus (arbovirus) vectors and the impact they might have in spreading pathogens in the New World, had not been asserted until the new millennium after the outbreak of the bluetongue disease (BTD) in Europe.

The first indications that midges function as arbovirus vectors were presented in 1944, when Du Toit discovered bluetongue virus (BTV) and African horse sickness virus (AHSV) (both family Reoviridae, genus *Orbivirus*) in field collected ceratopogonids of the genus *Culicoides* LATREILLE, 1809 in South Africa (Du Toit 1944). Further references of the involvement of *Culicoides* spp. in the spread of viral diseases were obtained within the following years, amongst others through the detection of BTV in *Culicoides variipennis* (COQUILLETT), 1901, *Culicoides imicola* KIEFFER, 1913, *Culicoides obsoletus* (MEIGEN), 1818 and *Culicoides puncticollis* (BECKER), 1903 (Price & Hardy 1954; Bowne & Jones 1966; Mellor et al. 1981, 1985; Jennings et al. 1983) and of AHSV in *C. variipennis* and *Culicoides nubeculosus* (MEIGEN), 1830 (Mellor et al. 1975, 1981).

In 1981, the transmission of the Oropouche virus (family Peribunyaviridae, genus *Orthobunyavirus*) from infected to susceptible hamsters by *Culicoides paraensis* (GOELDI), 1905 in laboratory trials succeeded (Pinheiro et al. 1981). While most of the viruses, which are transmitted by culicoid midges, are of veterinary importance, the Oropouche virus also infects humans and causes an acute febrile illness accompanied by common symptoms like headache, muscle and joint pain and skin rash, besides other occasional symptoms like anorexia, dizziness, chills, and photophobia. It is usually restricted to tropical regions of South and Central America though (Travassos da Rosa et al. 2017; Sakkas et al. 2018).

By the year 2000, more than 50 different viruses had already been isolated from the genus *Culicoides* alone, a ceratopogonid genus, which mainly consists of obligatory haematophagus species, sucking blood from mammals and birds (Mellor et al. 2000). 20 of these viruses belong to the family Peribunyiaviridae, 19 to Reoviridae and 11 to Rhabdoviridae (Meiswinkel et al. 1994).
But it was until the outbreak of the BTD in Central and Northern Europe in 2006, that the impact and danger of culicoid biting midges as arbovirus vectors became apparent and the research focused more on these nematoceran insects.

1.2 Bluetongue virus epidemic

Bluetongue virus (BTV) is an arthropod-transmitted, non-contagious, double-stranded RNA virus. Infecting domestic and wild ruminants and camelids, it causes a variety of symptoms like the name-giving erosions of oral mucosa, fever, oedema, salivation, lameness and apathy. Infection of pregnant hosts can cause abortions or serious malformations of the fetus (Elbers et al. 2008). At least 26 different serotypes of BTV exist (Maan et al. 2011), often going along with a variance in infectivity and severity for specific host species and breeds (Mellor et al. 2009). While the first observed hosts of this viral disease were cattle, sheep turned out to be more frequently infected and mostly stronger affected than other domestic or wild ruminants, often suffering lethal illness (Kampen 2008).

First descriptions of Bluetongue disease, back then called “Malarial Catarrhal Fever”, go back to the end of the 19th century in South Africa (Hutcheon 1893). By the 1920s, the disease had already spread to Europe. But prior to 1998, BTD appeared only occasionally in Europe and was restricted to few parts of the Mediterranean basin (Hendrickx 2009). As BTV was endemic mainly in tropic and sub-tropic climates, it commonly appeared between the latitudes of 40°N and 35°S (Gibbs & Greiner 1994). Due to biting midges of the genus *Culicoides* transmitting BTV almost exclusively, the spread of the virus was closely connected to the distribution area of its culicoid vector species.

Though Boorman pointed out the potential risk of BTD, an originally enzootic disease in the Mediterranean countries, threatening the European Economic Community based on the

presence of other potential vector species like *C. obsoletus* in this region (Boorman 1982), the main zone at risk was still believed to be the distribution area of C*. imicola* – an afrotropical midge species and the main vector of BTV in the old World (Hendrickx 2009; Kampen 2008). As this species' northern distribution boundary was believed to be the 40°N latitude (Jacquet et al. 2016) and as it had never been observed north of the alps until then, the risk of BTV epidemics in Northern and Central Europe was broadly considered as very unlikely. Therefore the BTV outbreak in Benelux, Germany and France in summer 2006 came as a total surprise for many.

The European epidemic of BTD started in July-August 2006 in the Maastricht area and rapidly spread all over Europe. It caused severe suffering to animals and high financial losses to the farming industry (Velthuis et al. 2011). The first appearance of BTV serotype 8 in Northern and Central Europe, combined with the presumed absence of *C. imicola* that far north, suggested, that other culicoid species might have been responsible for the transmission of the pathogen. Soon extensive research began to investigate the spread of BTV-8, its potential vector species, the distribution of its transmitters and possible introduction routes into Central and Northern Europe. National research, including monitoring programs, confirmed the absence of *C. imicola* in Germany, but was able to detect BTV in endemic culicoid species like *C. obsoletus*, *Culicoides scoticus* DOWNES AND KETTLE, 1952, *Culicoides dewulfi* GOETGHEBUER, 1936 and midges of the *Culicoides pulicaris* group (Meiswinkel et al. 2008; Mehlhorn et al. 2009a, b).

Hopes were expressed that the cold winter months and the end of the vector season might terminate the epidemic of the new pathogen in temperate regions. But contrary to expectations, the virus managed to persist, leading to new outbreaks of the same serotype the following years (Hoffmann et al. 2008). Only the introduction of an effective vaccination programme in 2008/2009 was able to stop the European BTV-8 epidemic (Baetza 2014). The following years, no infections were reported in Germany until December 2018 (Schulz et al. 2016). In other European countries though, new cases of BTD and different strains of BTV were reported within the following years after the first epidemic, leading "The Standing Committee on Vaccination in Veterinary Medicine" (STIKO Vet) to express a recommendation for vaccination of potential domestic host animals.

Illustrated by the example of BTV, the probability of future epidemics, caused by new pathogens, has been assessed as very likely. Only a few years after the European BTV-8 outbreak in Central Europe, this fearful prediction came true, when the never before observed pathogen Schmallenberg virus (SBV) (family Peribunyaviridae, genus *Orthobunyavirus*) emerged in Germany and neighbouring countries.

1.3 Schmallenberg virus epidemic in Europe

In late summer 2011, several cases of an undefined disease were reported from North Rhine-Westphalia, Germany, the Netherlands and Belgium in which several cattle showed a variety of unspecific symptoms like diarrhea, fever and a decrease in milk production, which disappeared after a few days (Hoffmann et al. 2012; Afonso et al. 2014). Early metagenome analyses revealed an unknown Shamonda-like virus, closely related to Akabane- and Aino-virus (both family Peribunyaviridae, genus *Orthobunyavirus*), to be the responsible agent (Hoffmann et al. 2012). As the first virus-positive samples originated from nearby the city Schmallenberg, the virus was named Schmallenberg virus (SBV). Soon it became apparent, that besides the mild clinic shown by adult hosts, SBV can lead to severe congenital defects in the offspring when susceptible, mother-animals are infected within a sensitive period during gestation (Garigliany et al. 2012a, b; Van den Brom et al. 2012). That way, the pathogen caused many abortions, stillbirths and malformations of newborn ruminants, which surfaced almost simultaneously with the detection of the new agent (Hoffmann et al. 2012).

Schmallenberg virus is an enveloped virus, which contains three segments of single-stranded negative sense RNA, a small (S), a medium (M) and a large (L) segment (Hoffmann et al. 2012). Based on its morphology it was soon classified to the family Bunyaviridae (Tarlinton et al. 2012). In 2019 though, the order Bunyavirales was taxonomically revised by the Committee on Taxonomy of Viruses (ICTV). Since then the genus *Orthobunyavirus* belongs to the newly established family Peribunyaviridae of the order Bunyavirales. Schmallenberg virus is a member of the Simbu serogroup, which contains at least 25 different viruses of the genus *Orthobunyavirus*. Due to the close relation between these viruses, many parallels

between SBV and other members of the Simbu serogroup exist. For example, foetal malformations, induced by viruses of the Simbu serogroup, comprise a variety of symptoms combined as the “arthrogryposis-hydranencephaly syndrome” (AHS), which can also develop after SBV infections of the mother animal during pregnancy. The most common damages induced by SBV are torticollis, arthrogryposis, cerebellar and narrow spinal cords, brachygnatia, kyphosis, scoliosis, hypoplasia of the central nervous system and poliomyelitis (Herder et al. 2012; Van den Brom et al. 2012; Peperkamp et al. 2015). As viruses of the Simbu serogroup mainly target the central nervous system, the phase of gestation at the time of viral infection is crucial and determines the severity of the developing AHS. While an early infection may lead to por- and hydrancephaly, an infection at a later phase of pregnancy might result in torticollis and arthrogryposis (Roth et al. 2013).

While none of the closest relatives of SBV causes disease in humans, the wider Simbu serogroup contains viruses with zoonotic potential, e.g. the Oropouche and the Iquito viruses (Aguilar et al. 2011). To gain certainty about the zoonotic potential of Schmallenberg virus, studies were conducted, examining people with high exposure to SBV. Soon the pathogen was classified as innocuous to humans with minimal zoonotic potential, though infections on a rare scale could not be ruled out (Tarlinton et al. 2012).

Following the infection of a ruminant with SBV, the viraemia is rather short and lasts only a few days (Hoffmann et al. 2012). Deriving from parallels of closely related viruses, it soon was assumed that the pathogen might induce a lifelong immunity in the host. Based on this hypothesis, two future outbreak scenarios had been considered as very likely:

theory one suggested a constant virus circulation on a low level, with only the susceptible new generations of domestic and wild ruminants being infected theory two forecasted the accumulation of susceptible hosts and a simultaneous absence of SBV. After several years, the high number of vulnerable host animals and the re-introduction of SBV into previously affected areas, could then lead to new epidemic outbreaks, nearly as strong as the first one in 2011/2012 (Méroc et al 2013; Veldhuis et al. 2015).

Transmission of SBV

Based on broadly available information about SBV's close relatives of the Simbu serogroup, e.g. Akabane virus, which is spread by mosquitos and biting midges in the Old World, it was assumed that Culicidae (Diptera) and Ceratopogonidae of the genus *Culicoides* could act as vectors for this pathogen in the New World, too (Hoffmann et al. 2012; Garigliany et al. 2012a). While the involvement of mosquitos could not be confirmed so far (Scholte et al. 2014; Wernike et al. 2014; Manley et al. 2015), SBV was soon detected within several midge species in different European countries (De Regge et al. 2012; Rasmussen et al. 2012; Elbers et al. 2013; Larska et al. 2013a, b; Kameke et al. 2016). Especially member species of the most frequent species complex, the Obsoletus Complex (including *C. obsoletus*, *C. scoticus*, *Culicoides chiopterus* (MEIGEN), 1830 and the isomorphic species *C. dewulfi*) were found to be infected with SBV. But also a few other culicoid species like *Culicoides pulicaris* (LINNAEUS), 1758, *Culicoides punctatus* (MEIGEN), 1804*, C. imicola and C. nubeculosus* tested positive for SBV, though the viral load was partly found to be on a sub-transmissible level (Rasmussen et al. 2012; De Regge et al. 2012; Elbers et al. 2013; Larska et al. 2013a, b; Balengien et al. 2014; Kameke et al. 2016).

SBV over the years

Time has shown that the number of SBV infected culicoid midges was highest during 2011, the first year of the SBV epidemic in Europe (Rasmussen et al. 2012; De Regge et al. 2012; Elbers et al. 2013). While the pathogen continued its spread throughout Europe, causing infections in livestock animals on a high level during 2012, rates of infected *Culicoides* vectors decreased rapidly after the first emergence of Schmallenberg virus within previously affected areas during consecutive years (De Regge et al. 2014; Elbers et al. 2015; Kameke et al. 2016). The decreasing numbers of infected midges and the high seroprevalences in European livestock consequently led to a low level of newly infected ruminants in 2013 (Wernike et al. 2015). During the next year (2014), but also during 2016, rising virus circulations during the vector seasons were detected (Wernike & Beer 2020).

The observations of the varying SBV circulations were also reflected in results of blood tests of young cattle, revealing the percentage of livestock, which had just overcome an SBV

infection. Yearly examinations of heifers conducted between winter 2012/13 until winter 2017/18, revealed, that the share of SBV seropositive animals differed strongly between years. It was highest in winter 2012/13 with almost 90% of cattle testing positive for SBV antibodies and lowest in 2014/15 and 2015/16. During the consecutive years, the number of seropositive animals increased again, so that around half of the tested heifers possessed antibodies against the pathogen in winter 2016/17 and 2017/18 (Landwirtschaftskammer Nordrhein-Westfalen 2020).

During 2017 and 2018, new cases of SBV infections in cattle and sheep were recorded on an almost regular basis (Wernike et al. 2018), but the extent of the virus circulation could not be assessed in detail (Wernike & Beer 2020). Apparently, the number of new infections remained relatively low with 33 reported cases in 2019 and (until august of) the following year 2020, 20 further cases of SBV infections were officially reported within Germany (Niedersächsiches Landesamt für Verbraucherschutz und Lebensmittelsicherheit 2020). Nevertheless, the actual number of Schmallenberg virus infections is assumed to be much higher than the reported and evident cases (Wernike & Beer 2020).

Contrary to early assumptions, an infection with SBV does not induce a lifelong immunity in the host. Investigations revealed that around 17% of previously seropositive cattle proved negative after only six years post infection. In addition, the within-herd seroprevalence decreased since 2011 due to the replacement of the older animals by susceptible younger ones (Wernike et al. 2018). Therefore, the risk of a new SBV outbreak, affecting new livestock generations as much as older animals, which have lost immunity, is growing steadily over time. It is still not conclusively clarified whether a steady virus circulation on a low level continues or if the accumulation of susceptible host animals might lead to another peak of an epidemic SBV outbreak in Europe in the future again.

Considering the reported cases of SBV-infections throughout the last years (2011 until 2020), data of seroprevalences of livestock animals and studies evaluating infection rates within biting midges, it was suggested, that the virus re-appears in larger waves approximately every three years (Wernike & Beer 2020).

Transmission routes

Besides the still highly unpredictable future of the pathogen, its origin and introduction routes into Europe in 2011 have not been clarified as well yet (Collins et al. 2019). Wind drift of culicoid vector species over long distances seems very likely, but also the import of insects along with infected animals as a reservoir host are only a few of the possible pathways to consider (Tarlinton et al. 2012). After its first emergence in late 2011 in Germany, the Netherlands and Belgium, SBV quickly spread throughout entire Europe. In 2012, in the Netherlands, a serosurvey was conducted to assess the total seroprevalence. It revealed that, solely during the 2011 epidemic, sheep, goat and cattle had been infected with SBV throughout the entire country (Veldhuis et al. 2013).

The quick spread of the epidemic into other European countries can be attributed to parallel means of distribution. The import/export of infected domestic animals (Larska et al. 2013a) and bovine semen (Hoffmann et al. 2013) are evident transmission routes. Also, the spread of arthropod vectors through anthropogenic pathways and natural factors such as wind drift, less the active flight behavior of *Culicoides*, seem to be the main distribution routes.

Even though the early suspicion that culicoid biting midges might act as arbovirus vectors for SBV could soon be confirmed, the abatement of these transmitting insects remained difficult as many aspects of their biology and behavior like choice of breeding sites, habitat preferences or overwintering strategies are still not well understood.

In case of completely new pathogens evolving in the future or diseases entering from other regions of the world, a useful pest management should be at hand though. Effective ways to reduce future epidemics could include a comprehensive vaccination programme and/or targeted vector control measures.

1.4 Pest control management

The probably most effective tools in regards to pest management are vaccination programs. At a downside, the development of vaccines, especially as a treatment of a completely new emerged pathogen, is time consuming and afterwards connected to additional costs for the

farmer. In spite of a possible interim decrease of virus circulation, many farmers might not be willing to infest into vaccines anymore and hence create the foundation for a new viral epidemic.

But beside vaccination programs, other methods of prophylactic control measures exist, e.g. the use of insecticides or repellents, administered as ear tags, strips or back rubbers for domestic animals.

Another approach within the field of pest management, are measures as part of a vector control strategy. The application of chemicals, e.g. insecticides like organophosphate compounds or insect growth regulators on the surface of developmental sites (Braverman 1994), was used to reduce the number of culicoid larvae as these mostly remain within the top soil layer. Besides creating subsequent resistances, this method turned out to be not effective (Borkent 2005). These days, the use of chemicals is also critically evaluated, especially in today's discussion about biodiversity and the strong decline of the insect fauna.

In case of culicoid biting midges, vector control strategies also comprise the attempt to prevent infection of pregnant ruminants by using gazed stables or to keep domestic animals indoors during times of high vector activity. While the use of gaze and nets often remained unrealizable due to farming management procedures, the temporal stabling of livestock revealed detriments as well. Most investigated *Culicoides* spp. were shown to not act strongly endophilic or exophilic, but to adjust their habitat preference based on outdoor temperatures and possibly other environmental factors (Baldet et al. 2008; Kameke et al. 2017). Another approach in preventing infections of pregnant ruminants addressed the rescheduling of the time of insemination for ruminant livestock, which was also discussed but dismissed as impractical.

A more long-termed attempt of vector reduction is to create vector-unfriendly landscape structures offering limited suitable breeding sites. This indeed is only feasible in specific areas, e.g. tourist-frequented regions, which feature culicoid nuisances on a local scale. Mostly, landscape modifications achieved by drainage of bogs, marshes or swamps, are said to be impractical (and too intruding into existing ecosystems), as pest species occur in such high numbers that a noticeable reduction is impossible (Borkent 2005). Furthermore, agriculturally dominated regions reveal many and repeatedly new created breeding

opportunities for farm-associated species. Therefore, a reduction of suitable larval developmental sites on farms seems insufficient.

To be able to conduct targeted vector control measures, a solid basis of information about the biology and ecological factors, which might influence the development and distribution of culicoid species and the species-specific breeding sites, is required. Even though the research has shifted its focus more onto this nematoceran insect family within the recent years, the ground knowledge is still far too small and especially concrete data on species-specific attributes is extremely scarce.

1.5 Biting midges – a general introduction

Ceratopogonidae are small dipteran insects, which measure 0.5-3.5 mm in size. They are holometabolous and undergo four distinct life stages: egg, larva, pupae and the adult stadium. After taking a blood meal, which is necessary for most culicoid species for egg development, the adult female lays her eggs into or onto a suitable breeding substrate. Days later, the larvae hatches and starts to feed on organic material. It develops through four instars before it pupates and the imago hatches. The development from egg to adult depends on surrounding conditions, in particular temperature, and can easily take two weeks or more. Adult midges can live up to 90 days under laboratory conditions, but are usually limited to 10-20 days under field conditions (Mellor et al. 2000).

In the Netherlands, more than half of the collected culicoid species were found to be either univoltine or bivoltine (Meiswinkel et al. 2013). That means they generate one or two generations per year. Other species, which include all BTV vectors, are multivoltine and can produce up to five or six generations per year depending on climate conditions (Meiswinkel et al. 2013).

Ceratopogonidae occur in all climatic regions and bigger landmasses of the world and appear even in altitudes of up to 4,000 meters above sea level (Mellor et al. 2000). Worldwide 6,267 extant ceratopogonid species are known to date, but the actual number is believed to be much higher. At present, known species are attributed to 133 genera, of which only four

include species sucking blood on vertebrates. These comprise *Austroconops* WIRTH & LEE, 1958, *Forcipomyia* MEIGEN, 1818 (subgenus *Lasiohelea* KIEFFER, 1921), *Leptoconops* SKUSE, 1889 and *Culicoides* LATREILLE, 1809 (Mellor et al. 2000). Among them, *Culicoides* is by now the best investigated genus, as it is the only one of real veterinary importance. That is due to the fact, that 96% of known culicoid species are haematophagous on vertebrates (Mellor et al. 2000) compared to only a few species within each of the other three mentioned genera. Currently 1368 culicoid species of 32 subgenera (Augot et al. 2017b) are described.

1.5.1 Blood-feeding of *Culicoides* spp.

The midges' species-specific choice of a blood host is not well understood at this time. While some species of the genus *Forcipomyia* are known to suck haemolymph from other insects like Meloidae, other foricpomyan midges prefer to take blood meals from mammals, birds, or frogs (Thompson 1969; Mullens et al. 1997). The range of potential blood hosts of *Culicoides* spp. is wide and includes vertebrates like humans, mammals, birds and amphibians (Lassen et al. 2010, also summarized in Augot et al. 2017a).

While some culicoid species show a strong and specific host preference, others feed on a wider range of hosts (Braverman 1994). *C. obsoletus* is known to be a generalist among the culicoid midges in many aspects including its blood host choices. It sucks blood on many different vertebrates like cattle, sheep, horse, donkey, bird, poultry, rabbit, mouse, roe deer, red deer, goat, wild cat and humans (Augot et al. 2017a). In comparison, *Culicoides albicans* (WINNERTZ), 1852 reveals to be a specialist and is only proven to suck blood on cattle (Elbers & Meiswinkel 2014).

Even though the range of potential blood hosts differs a lot between the various culicoid species, the mechanisms underlying the blood feeding act remain the same: as so called "pool feeders" biting midges use their strong piercing-sucking mouthparts to cut into the skin of their hosts and thereby damage small capillaries located inside the skin. The blood, emerging from the injured blood vessels, is being licked up by the insect. During the blood meal, which usually lasts 2-5 minutes (Borkent 2005), the Ceratopogonidae induces a salivary fluid to prevent coagulation and to keep the blood stream floating. This culicoid

saliva is known to cause painful dermal hypersensitivity responses (sweet itch) in equids and some sheep breeds (Anderson et al. 1991; Yeruham et al. 2000). Originating from the salivary glands from where it is injected into the host, it can also include viruses or other pathogens like bacteria, protozoa or nematodes (Linley 1985), causing an infection of the host animal.

The act of blood feeding is limited to female midges only as they require specific blood proteins for the maturation of fertilized eggs (oogenesis). The extent of necessary blood meals is also species dependent and results in different numbers of meals and varying volumes per meal.

The blood meal volume differs between species, too. While *C. imicola* sucks up to 0.139 µl, *Culicoides zuluensis* DE MEILLON, 1936 takes in about 0.410 µl blood (Braverman & Swanepoel 1981) and *C. variipennis* absorbs 0.56 mg (Tempelis & Nelson 1972). Just like the extent of the blood meals, the number of egg batches is also species-dependant and can reach a maximum of seven by a single female midge. *C. variipennis* for example can lay up to more than 1000 eggs divided in up to seven batches (Jones 1964).

Some culicoid species are able to lay their first batch of eggs without a precedent blood meal (autogeny), e.g. *Culicoides circumscriptus* KIEFFER, 1918 (Glukhova 1958), *Culicoides bermudensis* WILLIAMS, 1956 (Williams 1961), *Culicoides dendrophilus* AMOSOVA, 1957 (Amosova 1959) or *Culicoides melleus* COQUILLETT, 1901 (Koch & Axtell 1978).

The knowledge about the species property of autogeny is important for modelling approaches and vector control measures. The availability of a blood host might limit the distribution of a vector species. Therefore, autogeny is a tool to assure the conservation of a species even in the absence of potential blood hosts. It also reduces mortality in the first gonotrophic cycle. This seems especially important, if breeding sites of culicoid species follow a patchy distribution (Kettle 1977).

1.5.2 Egg stadium

The eggs of biting midges are usually laid “singly, in scattered loose groups, or in strings and masses with a gelatinous coating” (Szadziewski et al. 1997). They usually measure between 250-500 µm (Wirth & Blanton 1974; Braverman 1994), are banana shaped and white when newly laid, but darkening to brown (Wirth & Blanton 1974). While the number and size of laid eggs can vary, depending on the amount and quality of the blood meal (Kettle 1962), a single batch of eggs may include up to 250 eggs and can result in total egg numbers of more than 1100 per female (Wirth & Blanton 1974).

The substrates for egg deposition are species-specific and comprehend an extremely wide variety of biotopes and different soil factors. As female midges prefer egg deposition in company, local accumulations of egg batches may occur, which result in a high number of empty emergence trap catches (Dove et al. 1932) during research activities. This indeed hinders necessary research and impedes the much needed identification of the species-specific choice of breeding sites for culicoid midge species.

1.5.3 Breeding substrates

Eggs of aquatic breeding species are laid directly on the water surface of still lakes or streams or onto other water accumulations such as tree holes. Species of the genus *Culicoides* belong to terrestrial or semi-aquatic breeders, which usually deposit their eggs on a moist substrate, e.g. wet leafs, humid soil or decaying organic matter. This is mainly due to the fact that eggs of biting midges are susceptible to desiccation and therefore need a high level of humidity (Kettle 1977).

Even though an ecological classification into aquatic, semi-aquatic and terrestrial forms is broadly accepted, it is imprecise as many species inhabit a wide range of habitats, comprising huge variances of soil conditions (Szadziewski et al. 1997; Kameke et al. 2021). Nevertheless, the genus *Culicoides* is considered to contain mostly semi-aquatic species, which require larval substrates with a high level of humidity (Kettle 1962). Also, a high level of organic matter is often assumed to determine the potential of a substrate as a larval

developmental site (Kettle 1962). Following these imprecise specifications, several studies were conducted to assess larval habitats of culicoid biting midge species. Most of these investigations had a strong focus on potential developmental sites located on farms though, as these play a big role when it comes to epidemic outbreaks and vector control measures. Among the common farm-breeding species are *C. obsoletus*, *C. scoticus*, *C. chiopterus* and *C. dewulfi.* But also members of the Pulicaris Complex like *C. pulicaris* sensu strictu can be found on farms, too (Zimmer et al. 2008), as can be closely related species, e.g. *C. punctatus*, *C. impunctatus* GOETGHEBUER, 1920 from time to time (Zimmer et al. 2013). Much less well investigated are natural biotopes, also serving as emergence sites of *Culicoides* spp. These habitats can represent good developmental conditions for a broad variety of species, which usually appear in smaller numbers than farm breeding species (Kameke et al. 2021).

Nevertheless, the overall knowledge in this field is still scarce, especially in regards to collecting concrete data of physicochemical soil factors, which might determine and limit the species' choice of developmental substrates. The identification of breeding sites for most *Culicoides* spp., which do not produce high numbers of specimens, is also still missing.

For a successful and targeted treatment of larval and pupal habitats as part of a vector control measurement, it is inevitable to know the potential breeding sites of different midge species. As different soil factors enhance or inhibit the quality as a potential breeding substrate, they might therefore be crucial in understanding the midges' choice. At the same time, rarely occurring midge species that have no impact as arbovirus vectors, should be protected. In this context, a solid basis of information about the co-existences of different culicoid species is also beneficial.

1.5.4 Larval development & pupae

After at least 2 to 7 days under favorable conditions, the first of four instars hatches and starts feeding on organic matter. While some midge species prefer decaying leafs, rotten plants, funghi, algae (Kampen & Kiel 2006), other species are predatory and devour nematodes, rotifers or other invertebrates (Kettle et al. 1975; Mullen & Hribar 1988; Clark & Fukuda 1967). Although bibliographical references are not clear, they mostly describe the

third and fourth larval stage as the hibernating stadium in temperature climates (Szadskiewski 1997; Borkent 2005; Kampen & Kiel 2006). Only a few species are known to hibernate as eggs (Borkent 2005). Due to a possible diapause and overwintering, the duration of an entire life cycle can last between 14 days to over a year, depending on the species, its species-specific life cycle and climate. The overwintering larvae alone can live as long as 7-9 month. In the following spring and early summer, the emerging midge fauna is therefore commonly more diverse than during the later summer period (Borkent 2005, Kameke et al. 2021).

The larvae of *Culicoides* spp. are apneustic and depend on cutaneous respiration. Therefore, they avoid anaerobic habitats (Kettle 1977). As mobility is reduced during the larval stage, their habitats mainly result from selection by the ovipositing female. Nevertheless, larvae were found to avoid unfavorable environmental conditions by moving vertically within their substrate (Blackwell & King 1997; Mullens 1992). Due to the higher levels of oxygen within the upper layers of soil, most culicoid larvae remain within the top 5-10 cm of their substrate (Uslu & Dik 2006). To assess the midges' species-specific choice of breeding sites, the use of emergence traps offers the most reliable results even though the number of collected individuals is usually very low.

The entire duration of larval development is strongly dependent on environmental factors such as temperature or availability of food and may take up 20-25 days at optimal temperatures of 20-25 °C (Gutsevich 1973) before pupation begins. Most ceratopogonid larvae pupate near the surface of their developmental substrate. Compared to the larval stage, the pupae of terrestrial species, possesses two pairs of respiratory organs and can therefore obtain air (Borkent 2005). After approximately 3-5 days of pupation, the adult midge hatches.

1.5.5 The adult midge

In general, the emergence of males begins slightly earlier than that of female midges, but for most of the time, takes place simultaneously. Directly after hatching, midges usually rest

briefly and then take off to another resting place for their cuticle to finish hardening (Borkent 2005).

Males of few midge species fly around singly searching for a mate, while male specimens of most species form swarms. Females, ready to mate, fly through these swarms and are being grabbed by an attracted male. Copulation can take place during flight or after landing on a substrate. After copulation, most female *Culicoides* require a blood meal for oogenesis and therefore start to seek out for a suitable host. Depending on the availability of a blood host, the female might fly bigger distances of up to 4 km, but usually remains close to her emergence site. After feeding, the development of eggs takes a few days, in which the female midge searches for a suitable substrate to lay her eggs onto. Specimens of both sexes feed on nectar or honeydew, if they fly over longer distances and are in need of additional energy. Therefore, this usually applies to longer lived ceratopogonid species (Borkent 2005).

Phenology

Some of the European *Culicoides* like *C. obsoletus* produce several generations per year (multivoltine), others appear to be univoltine with only one generation. As the duration of each life cycle and the number of generations per year strongly depend on meteorological conditions, these species-specific attributes differ between climatic regions and a clear distinction is often difficult to make (Szadziewski et al. 1997). The phenology of culicoid biting midges in Northern and Central Europe reveals two peaks of adult midge activity over the year. The spring peak usually takes place in May and June when the overwintering midge larvae finish their development and the imagines hatch. The second population peak can often be observed during late summer, when adults of the second generation emerge. Univoltine species instead, commonly appear after the spring peak and remain active until August. Once temperatures decrease, the numbers of midges commonly declines, too. Depending on weather and climatic conditions, active midges might disappear from October on. Some of the last active midge species during the year are *C. obsoletus*, *C. grisescens, C. fascipennis* and *C. punctatus* (Szadziewski 1997; Kameke et al. 2021).

Contrary to this finding, a study conducted within Germany revealed low midge activity between April and May, which increased constantly until October, when mostly midges of the Obsoletus Complex were sampled in UV-light traps (Mehlhorn et al. 2009b). In this case, more culicoid specimens were still active by the end of the year than in early spring. It also became evident, that adult midges of the Obsoletus Complex appeared inside stables during (warm) winter months and that even in temperate regions, no real vector-free period seems to exist (Mehlhorn et al. 2009a, b).

1.5.6 Overwintering of culicoid biting midges

Before the detection of midge activity during mild winter days (Mehlhorn et al. 2009a, b; Hoffmann et al. 2009), it was believed that all *Culicoides* spp. would hibernate solely in a developing stage. While multivoltine species were said to diapause as third or fourth instar larvae, univoltine species were believed to survive the cold months as eggs (Mellor et al. 2000; Rawlings & Mellor 1994; Rowley 1967; Szadziewski 1997). Therefore, the unexpected re-appearance of BTV in its second year raised questions about the overwintering mechanisms of the pathogen and its vectors. But even after the measured culicoid flight activity during winter, some doubt remained, whether it was the low midge population during the cold months that kept the virus circulation alive (Napp et al. 2011). Surely that finding would explain one of the possible mechanisms underlying re-emergences of viral diseases in consecutive years and might also explain the acute infection of SBV in sheep during winter 2013 in Germany (Wernike et al. 2013). On the contrary, other studies did not measure any culicoid activity inside or outside stables for a longer period during winter time (Kameke et al. 2017). That illustrates that especially in colder regions or during cold winters, even stables do not always represent good conditions for culicoid midge activity. The presence of *Culicoides* depends on temperatures inside the stables, which are (among other factors) determined by the type of livestock animals being kept indoors. And while single midges can still be found during days with short-termed minimum temperatures under minus one degree, the species-specific threshold temperatures starting culicoid activity is seven degree and higher (Kameke et al. 2017).

These results illustrate the need for additional comprehensive research, as a solid foundation of knowledge is necessary in order to estimate a pathogen's potential and likelihood to overwinter during an outbreak scenario.

1.6 Objectives

The importance of culicoid biting midges as arbovirus vectors became apparent after the outbreak of BTV-8 in Europe in 2006. After the emergence of the Schmallenberg virus in Europe in late 2011, several European countries began immediate investigation to search for its potential vector species. Soon, first studies were able to detect the pathogen inside different *Culicoides* spp. (e.g. Rasmussen et al. 2012; Elbers et al. 2013; De Regge et al. 2012). The virus was able to spread across Europe in record time, causing suffering to affected animals and high financial losses to the farming industry. Once again, the lack of basic information regarding this dipteran family became obvious.

In order to establish epidemic outbreak prognosis models and targeted vector control measures, it is crucial to understand the biology, behavior and dispersion of the vectors. Even more so, as the distribution and spread of SBV are strongly dependent on the availability of susceptible host animals and the presence of its arthropod vectors, culicoid biting midges. As the presence of midge species is highly determined by the disposability of suitable breeding substrates, a solid foundation about the species-specific breeding sites is necessary. Overwintering mechanisms of the virus are closely related to the hibernation strategies of its vectors and might also play a critical role in the ability of a pathogen to overdue the cold winter months.

The object of the present dissertation work was to determine the level of virus circulation within culicoid biting midges, illustrating their impact on the spread of SBV in Germany. Furthermore, the question of overwintering strategies was addressed: the existence of a vector-free period during winter, threshold temperatures triggering culicoid activity in early spring and the influence of livestock on the present midge fauna were investigated. The composition of *Culicoides* midges during summer was analyzed in regards to their chosen breeding sites, their phenology and the co-existence of culicoid species.

Therefore, the following studies were conducted:

The examination of the involvement of *Culicoides* spp. in the German spread of SBV after its first emergence in 2011, which resulted in the publication
Kameke, D., Werner, D., Hoffmann, B., Lutz, W., Kampen, H. (2016): Schmallenberg virus in Germany 2011-2014: searching for the vectors. Parasitology Research, 115: 527-534 (chapter 2.1).

A determination of the winter activity of culicoid biting midges and their participation as viral vectors during an acute case of SBV-infection of sheep in Germany, 2013. The results of this project were published under
Wernike, K., Kohn, M., Conraths, F.J., Werner, D., Kameke, D., Hechinger, S., Kampen, H., Beer, M. (2013): Transmission of Schmallenberg virus during Winter, Germany. Emerging Infectious Diseases, 19: 1701-1703
(chapter 2.2).

An investigation of the presence of *Culicoides* spp. during the winter months, evaluating the influence of livestock animals on the endo- and exophilic flight activity of midges, which was published under
Kameke, D., Kampen, H., Walther, D. (2017): Activity of *Culicoides* spp. (Diptera: Ceratopogonidae) inside and outside of livestock stables in late winter and spring. Parasitology Research, 116: 881-889.
(chapter 2.3).

The identification of culicoid breeding sites in agriculturally used and natural habitats in Germany with a further analysis of the phenology of sampled *Culicoides* spp. and co-existing species available under
Kameke, D., Kampen, H., Wacker, A., Werner, D. (2021): Field studies on breeding sites of *Culicoides* LATREILLE (Diptera: Ceratopogonidae) in agriculturally used and natural habitats. Scientific Reports, 11: 10007.
(chapter 2.4).

2. Results

Part of this doctoral thesis have been published in peer-reviewed journals:

Kameke, D., Werner, D., Hoffmann, B., Lutz, W., Kampen, H. (2016): Schmallenberg virus in Germany 2011-2014: searching for the vectors. Parasitology Research, 115: 527-534.

I was the main conductor of this project. I was involved in the collection and identification of the Ceratopogonidae. I was also the main contributor to data analysis and writing of the manuscript.

Wernike, K., Kohn, M., Conraths, F.J., Werner, D., **Kameke, D.**, Hechinger, S., Kampen, H., Beer, M. (2013): Transmission of Schmallenberg virus during Winter, Germany. Emerging Infectious Diseases, 19: 1701-1703.

I was involved in the collection and identification of the insects and provided additional data about proceedings and surrounding conditions.

Kameke, D., Kampen, H., Walther, D. (2017): Activity of *Culicoides* spp. (Diptera: Ceratopogonidae) inside and outside of livestock stables in late winter and spring. Parasitology Research, 116: 881-889.

I was the main conductor in the collection and identification of biting midges, data analysis and writing of the manuscript.

Kameke, D., Kampen, H., Wacker, A., Werner, D. (2021): Field studies on breeding sites of *Culicoides* LATREILLE (Diptera: Ceratopogonidae) in agriculturally used and natural habitats. Scientific Reports, 11: 10007.

I was the main conductor in the collection and identification of biting midges, data analysis and writing of the manuscript.

Daniela Kameke Prof. Dr. Alexander Wacker

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ORIGINAL PAPER

Schmallenberg virus in Germany 2011–2014: searching for the vectors

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Abstract Following the emergence of Schmallenberg virus (SBV) in 2011, 21,397 culicoid biting midges (Diptera: Ceratopogonidae) from targeted and non-targeted sampling activities carried out during the summer months of 2011 to 2013 and in late 2014 in various regions in Germany were analyzed for the virus by real-time RT-PCR. While no SBV was found in biting midges collected during 2011 and 2013, 2 out of 334 pools including 20 and 22 non-engorged females of the Obsoletus complex sampled in 2012 tested positive for the SBV S-segment with C_t values of 42.46 and 35.45. In addition, 673 black flies (Diptera: Simuliidae) captured during the same studies were screened for the presence of SBV and proved negative. In late autumn 2014, biting midges were collected again in a limited study in eastern Germany after some cases of SBV infection had occurred in a quarantine station for cattle. Due to the unfavorable seasonal weather conditions, only few specimens were caught, and these were also negative for SBV. The German experience suggests that biting midge collections launched only after an outbreak and are not locally targeted may be ineffective as to virus detection. It rather might be advisable to collect biting midges at sentinel farms on a permanent basis so to have material available to be examined in the case of a disease outbreak.

Keywords SBV · Ceratopogonidae · *Culicoides* · Biting midges · Obsoletus complex · Simuliidae

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Introduction

In late summer 2011, Schmallenberg virus (SBV), a new Orthobunyavirus (family Bunyaviridae) emerged in Germany. SBV infects ruminants and causes a mild clinic with possible symptoms such as diarrhea, fever, or a decrease in milk production. Infection of pregnant animals can lead to miscarriages, stillbirths, or severe deformations of the unborn (Hoffmann et al. 2012). Once infected, hosts are believed to develop immunity (Conraths et al. 2013a). A zoonotic potential seems unlikely but could not be completely ruled out (Ducomble et al. 2012; Reusken et al. 2012). Based on the close relationship of SBV to well-known viruses such as the Akabane virus (family Bunyaviridae, genus Orthobunyavirus), which is widely distributed in Africa and Asia and transmitted by Ceratopogonidae and Culicidae, it was hypothesized that these groups of arthropods could also function as possible vectors of SBV (Hoffmann et al. 2012; Garigliany et al. 2012). While studies could not confirm the involvement of mosquitoes (Scholte et al. 2014; Wernike et al. 2014; Manley et al. 2015), SBV was soon found within culicoid biting midges of the Obsoletus complex (Rasmussen et al. 2012). Further studies confirmed these findings and detected the virus in *Culicoides obsoletus* (Meigen), 1818; *C. chiopterus* (Meigen), 1830; *C. dewulfi* Goetghebuer, 1936; *C. scoticus* Downes & Kettle, 1952; *C. punctatus* (Meigen), 1804; *C. pulicaris* (Linnaeus), 1758; *C. nubeculosus* (Meigen), 1830; and *C. imicola*

Kieffer, 1913 (e.g. De Regge et al. 2012, 2014; Elbers et al. 2013; Larska et al. 2013a, 2013b; Balenghien et al. 2014).

Additionally, specimens belonging to laboratory colonies of *C. nubeculosus* and the North American species *C. sonorensis* Wirth & Jones, 1957 were found to reproduce the virus upon feeding on a viraemic blood source and facilitate dissemination into the salivary glands under experimental conditions (Veronesi et al. 2013).

While most SBV-infected midges were collected between early August and late October (De Regge et al. 2012, 2014; Rasmussen et al. 2012, 2014; Elbers et al. 2013; Larska et al. 2013a, 2013b), Elbers et al. (2015) detected the virus in 2012 in culicoids sampled already in July. Probably due to the Mediterranean climate, SBV was furthermore present in catches made in Italy as early as May suggesting the likelihood of SBV overwintering in midges (Goffredo et al. 2013) and as late as November (Goffredo et al. 2013; Balenghien et al. 2014).

The involvement of black flies (Simuliidae) in the transmission of SBV has never been investigated before. Simuliids are closely related to the Ceratopogonidae and include known vectors of nematodes. By contrast, only few viruses have been detected in Simuliidae so far (Braverman 1994; Smith et al. 2009), which might be attributed to the scarce number of studies conducted. Therefore, an involvement of simuliids in the transmission of SBV cannot be ruled out. Also, the rapid geographic spread of SBV in 2011 and 2012 indicated that arthropods other than Ceratopogonidae might have been involved in the transmission of the pathogen (Goffredo et al. 2013).

Several European countries were affected by SBV during its first transmission season in 2011 (Conraths et al. 2013a). During the vector season in 2012, the disease re-emerged in countries already affected and continued to spread to other yet uninvolved European countries (Conraths et al. 2013b). In 2013, the number of new SBV infections decreased significantly in countries previously affected, a tendency that continued in 2014.

In Germany, SBV activity had its peak in 2012. Southern and eastern regions of the country were much less affected at that time than western, central, and northern regions (Fig. 1). During 2013, the number of new infections decreased significantly, and the main viral activity took place in southern Germany (federal state of Bavaria). In 2014, only a handful of new cases were registered at the beginning of the year, and SBV seemed to have disappeared. However, in October 2014, new cases emerged in several German localities (Wernike et al. 2015). Some of them were related to an open quarantine station near the city of Cottbus, eastern Germany, where several cattle were proven to be freshly infected by SBV.

The objectives of this paper were I) to analyze the level of SBV circulation in culicoid biting midges in Germany for 2011, 2012 and 2013; II) to identify potential SBV vector species in Germany; and III) to check for the presence of SBV-infected culicoid biting midges near a single stable with acute viral activity in late 2014.

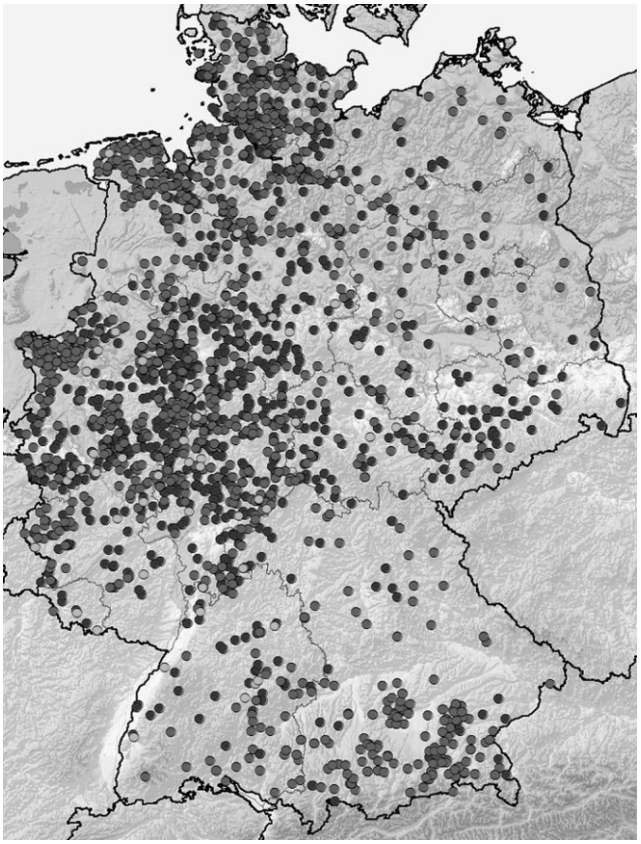

Fig. 1 Reported cases of SBV infection in Germany as of 22 January 2013. *Dark-gray dots*: cattle, *black dots*: sheep, *light gray dots*: goats (source: FLI Archive. Maps of the distribution of 'Schmallenberg virus' in Germany, www.fli.bund.de/no_cache/de/startseite/aktuelles/tierseuchengeschehen/schmallenberg-virus/archiv-der-karten-2013.html)

Materials and methods

Collection and identification of culicoid biting midges

During the summer months of the years 2011, 2012, and 2013, regular insect collections were done using BG sentinel UV-light suction traps (BG-S) and Onderstepoort Veterinary Institute UV-light suction traps (OVI traps).

In 2011 and 2012, the collections were not specifically targeted at ceratopogonids or simuliids as potential vectors of disease agents and only some of the traps were operated inside, or in close proximity to, animal shelters harboring potential infective blood hosts such as cattle, sheep, or goats. By contrast, most of the traps operated in 2013 were set up on farms keeping ruminants. Two insect traps were installed for several weeks close to a quarantine stable for cattle soon after the occurrence of acute SBV infections in 2014. All traps

(Fig. 2) were run once a week for approximately 24 h unless stated otherwise.

2011 and 2012, BG-S traps were operated between April 21 and October 22, 2011 and between May 20 and November 3, 2012 at 10 collection sites per year. As three sites remained identical over the two years, 17 sites were sampled altogether. The distribution of the collection sites throughout Germany was as follows: nine in western Germany, one in central Germany, two in southern Germany, one in eastern Germany, two in north-eastern Germany, and two in the northern parts of the country (Fig. 2).

2013, BG-S and OVI traps were operated between April 19 and October 25 at 22 collection sites throughout Germany. Nineteen sampling sites were located in the southern, eastern, and north-eastern parts of Germany, two sampling sites in western and one sampling site in northern Germany (Fig. 2).Three of the locations had already been utilized in at least one of the previous years. During the last week of January, three additional BG-S were set up inside a sheep stable with fresh cases of acute SBV infections (Wernike et al. 2013).

2014, Two OVI traps were operated between November 7 and December 18 at two collection sites near a quarantine stable with acute cases of SBV infection in cattle (approximately 40 km southeast of Cottbus, close to the Polish border) (Fig. 2). Both OVI traps were run on a daily basis.

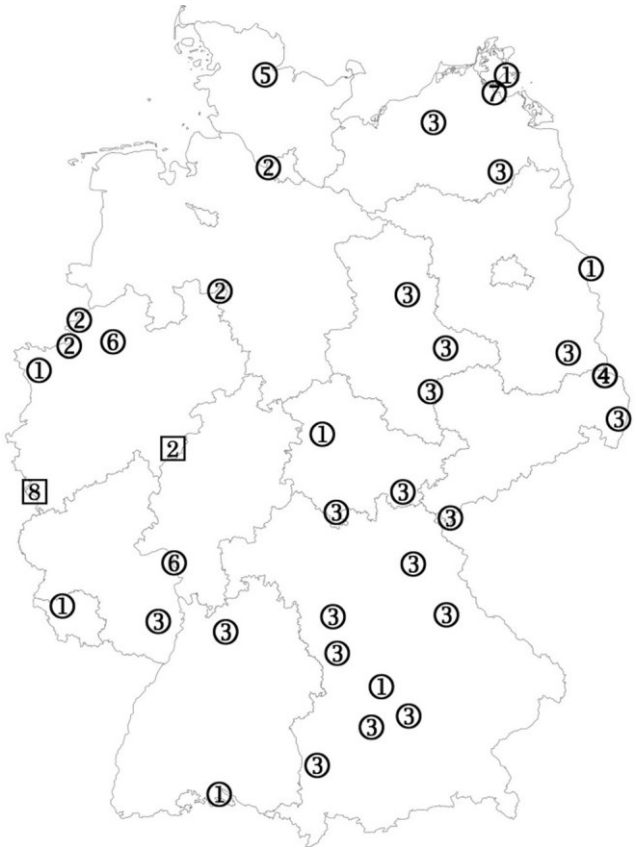

Fig. 2 Collection sites and SBV findings during the various collection years. *1*, 2011; *2*, 2012; *3*, 2013; *4*, 2014; *5*, 2011–2013; *6*, 2011+2012; *7*, 2011+2013; *8*, 2012+2013; negative for SBV (*enclosed in circle*), positive for SBV (*enclosed in square*)

All insects were collected in 75 % ethanol until further investigation under a stereo microscope (Olympus SX12). Based on their wing maculation, female culicoids were morphologically identified to complex or species level following the keys of Delécolle (1985) and Mathieu et al. (2012). In this paper, references to the ʙObsoletus complex^ include the species *C. obsoletus* s.s., *C. scoticus*, *C. chiopterus*, and the isomorphic species *C. dewulfi*.

Insect pools

For virus detection, female *Culicoides* Latreille, 1809 were pooled according to species or species complex as well as collection site and date, with up to 50 specimens per pool. Blood-fed individuals were pooled separately following the same criteria.

Additionally, Simuliidae were pooled according to sampling site and date but without further identification. Pool sizes ranged from 1 to 25 specimens. Again, blood-fed individuals were pooled separately following the same criteria.

Detection of SBV genome

Viral RNA was extracted using the NucleoSpin 96 Virus Core Kit (Macherey-Nagel) following the manufacturer's instructions. Eluates were then tested by the SBV-S3 one-step real-time RT-PCR as described by Bilk et al. (2012) and Hoffmann et al. (2012). As part of a duplex RT-qPCR, the assay was combined with internal control assays: a universal internal control system IC2-RNA (Hoffmann et al. 2006) and the co-amplification of a housekeeping gene, beta-actin (Toussaint et al. 2007).

Weak positive results with C_t values over 35 were retested to confirm positivity of the samples. Retesting was performed with the S3- and the L1.4-RT-qPCR assay (Fischer et al. 2013). If both assays showed positive results, the pool was considered SBV-positive. If the less sensitive L-amplicon could not be detected, the retest was repeated following a new extraction. The sample was still considered positive if the S3-amplicon could be detected in every(re)test.

Results

A total of 21,397 culicoid biting midges from 171 samples were divided into 945 species-specific pools (average pool size=22.64 specimens), including 110 pools containing 2110 blood-fed individuals (Table 1). While 19,107 of the midges belonged to the Obsoletus complex, the most abundant

Table 1 Species/species group and numbers of female *Culicoides* and Simuliidae specimens and species composition tested for SBV including number of examined pools (in parentheses) and blood-fed individuals (in bold)

Year	*Culicoides*	Determined as ʙObsoletus complex^	Determined as ʙPulicaris complex^	Specimens identified to species level	Identified species [numbers of specimens]	Simuliidae
2011	4999 (218)	4042 (105)	952 (110)	5 (3)	*C. achrayi* Kettle & Lawson, 1955 [3]	633 (37)
	32 (12)	22 (6)	5 (3)	5 (3)	*C. circumscriptus* Kieffer, 1918 [1]	0
					C. riethi Kieffer, 1914 [1]	
2012	5562 (334)	5197 (265)	327 (53)	38 (16)	*C. achrayi* Kettle & Lawson, 1955 [4]	3 (2)
	72 (22)	54 (9)	5 (3)	13 (10)	*C. clastrieri* Callot, Kremer & Deduit, 1962 [2]	2 (1)
					C. grisescens Edwards, 1939 [19]	
					C. impunctatus Goetghebuer, 1920 [1]	
					C. newsteadi Austen, 1921 [5]	
					C. picturatus Kremer & Deduit, 1961 [1]	
					C. salinarius Kieffer, 1914 [1]	
					C. segnis Campbell & Pelham-Clinton, 1960 [2]	
					C. truncorum Edwards, 1939 [3]	
2013	10,803 (385)	9836 (243)	858 (118)	109 (24)	*C. achrayi* Kettle & Lawson, 1955 [9]	37 (9)
	2006 (76)	1973 (62)	21 (8)	12 (6)	*C. grisescens* Edwards, 1939 [7]	4 (2)
					C. impunctatus Goetghebuer, 1920 [5]	
					C. newsteadi Austen, 1921 [85, 1]	
					C. picturatus Kremer & Deduit, 1961 [1]	
					C. vexans (Staeger), 1839 [2]	
					C. obsoletus (Meigen), 1818 [1][a]	
2014	33 (8)	32 (7)	0	1 (1)	*C. punctatus* (Meigen), 1804	0
	0	0	0	0		0
Total	21,397 (945)	19,107 (620)	2137 (281)	153 (44)		673 (48)
	2110 (110)	2049 (77)	31 (14)	30 (19)		6 (3)

[a] Described by Wernike et al. (2013)

species complex, 2261 individuals were members of the Pulicaris complex and 29 specimens belonged to other culicoid species.

Additionally, 673 Simuliidae from 30 samples, predominantly collected between May and September 2011 (Table 2), were sorted and pooled as described above, creating 48 pools. Six of the black flies were blood-fed (Table 1).

Among the 945 culicoid pools screened for SBV, two pools (0.2 %) were weakly positive for the pathogen. Both positive samples were collected in 2012 in western Germany, resulting in an average of 0.6 % (2/334) of positive pools for 2012. No *Culicoides* sampled during 2011, 2013 and 2014, and none of the Simuliidae tested positive for SBV.

Positive pool 1: The pool contained 20 specimens of the Obsoletus complex which had been sampled on September 3, 2012 in the German federal state of North Rhine-Westphalia (N 50° 57′ 40″, E 8° 19′ 16″) inside a barn housing a few sheep and goats. The pool showed a C_t value of 42.46 for the S3 region (positive control C_t=27.23).

Retest C_t values were 37.36 and 38.23 (mean value= 37.795) for the S3-RT-qPCR and 36.96 and 37.41 (mean value=37.185) for the L1.4-RT-qPCR.

Positive pool 2: The pool contained 22 specimens of the Obsoletus complex which had been sampled on August 29, 2012 in North Rhine-Westphalia (N 50° 31′ 55″, E 6° 19′ 21″) at a forester's lodge. The pool showed a C_t value of 35.45 for the S3-RT-qPCR (positive control C_t=27.07), and the retests delivered C_t values of 34.86 and 37.14 (mean value=36). The L1.4-RT-qPCR reacted negative in all tests. The retest including re-extraction confirmed previous results with C_t values of 38.90 and 38.28 (mean value= 38.59) for the SBV-S3-RT-qPCR and negative results for the L1.4 assay.

Discussion

In this study, more than 21,000 female culicoid biting midges collected between 2011 and 2014 were screened for SBV. Only 0.2 % of the tested pools were weakly positive for the pathogen which is a remarkably low percentage considering the high SBV activity in ruminants in 2011 and 2012 and compared to studies in adjacent countries like Denmark (Rasmussen et al. 2012, 2014), the Netherlands (Elbers et al. 2013, 2015), Belgium (De Regge et al. 2012, 2014), and Poland (Larska et al. 2013a, 2013b). A more detailed analysis of each year of collection revealed that in our study, no SBV-positive biting midge was caught in 2011 when SBV was discovered. Since all hosts must have been susceptible to the virus at that time, a relatively high level of virus circulation should have been assumed soon after the outbreak. Especially, due to the majority of the tested ceratopogonids of our study (n=4130) being collected during the months of July–October 2011 (Table 2), the presumed peak season of transmission, a high chance of detecting positive midges was expected for

Table 2 Sampling periods of *Culicoides* and Simuliidae tested for the presence of SBV

Year	2011		2012		2013		2014	
Month	*Culicoides*	Simuliidae	*Culicoides*	Simuliidae	*Culicoides*	Simuliidae	*Culicoides*	Simuliidae
January	n.d.	n.d.	n.d.	n.d.	1	0	n.d.	n.d.
April	137	0	n.d.	n.d.	917	0	n.d.	n.d.
May	331	189	1166	0	2486	3	n.d.	n.d.
June	401	112	667	1	2334	3	n.d.	n.d.
July	489	50	914	2	3159	31	n.d.	n.d.
August	2648	133	1822	0	1356	0	n.d.	n.d.
September	988	120	487	0	274	0	n.d.	n.d.
October	5	29	492	0	276	0	n.d.	n.d.
November	n.d.	n.d.	14	0	n.d.	n.d.	33	0
Total	4999	633	5562	3	10,803	37	33	0

n.d. not done

2011. This is especially true, as most of the insect traps were located in western Germany where the first cases of Schmallenberg disease had been observed. However, the assumed high level of virus circulation is not reflected in the number of *Culicoides* positive for SBV in the present study. As the sensitivity of RNA extraction and amplification with the used protocol was demonstrated previously (Hoffmann et al. 2009), a methodological reason is not considered plausible. A degradation of viral RNA by the time of processing to explain the lack of SBV-positive midges for 2011 in Germany is also rather unlikely since the insects were stored in 75 % ethanol and were in good optical condition when tested. It seems more reasonable to assume that the choice of collection sites is the main factor for the lack of SBV-infected midges captured. As all insect traps operated during 2011 and 2012 were part of a monitoring program with a different focus, many of them were not installed right on farms with blood hosts susceptible to SBV. This finding is in accordance with Rasmussen et al. (2014) where none of the midges, collected during 2011 at various untargeted sampling sites in Denmark, tested positive for SBV, whereas a previous Danish study showed infection rates of 9.1 % at only a few targeted collection sites sampled during the same year (Rasmussen et al. 2012). Since the flight radius of ceratopogonids is limited, with 2–3 km at most (Kluiters et al. 2015), the presence of potential blood hosts might be crucial in finding the pathogen and might therefore explain the lack of SBV-positive specimens.

In the Netherlands, Elbers et al. (2013) were able to find the virus in biting midges in 2011 with a relatively low percentage of infected pools (2.3 %=14/610 pools, with 10 midges/pool). By contrast, other studies carried out in 2011 showed much higher percentages of positive *Culicoides* midges. While Rasmussen et al. (2012) found a mean value of 9.1 % (2/22 pools, with 5 midges/pool) of SBV-infected midges in Denmark, infection prevalences varied locally between 3.7 % (5/134 pools, mean pool size=8.3 midges/pool) and 15.9 % (7/44 pools, mean pools size=19.3 midges/pool), with a mean infection prevalence of 6.7 % (12/178 pools) in Belgium (De Regge et al. 2012).

Our study confirms the circulation of SBV in German *Culicoides* for 2012, when the disease had its peak in countries previously affected by the pathogen. The two positive pools in Germany in 2012 demonstrated SBV in females of the Obsoletus complex but failed to confirm other species/species groups to carry the virus or to add new species to the list of potential vectors. The C_t values of the SBV S-segments in both positive samples were rather high, 42.46 and 35.45, respectively. Both Larska et al. (2013b) and De Regge et al. (2014) presented bimodal distributions of C_t values which are believed to display SBV at a transmissible and sub-transmissible level. The high C_t values in our study suggest that both positive midges did not contain SBV at transmissible levels and that the virus did not replicate inside the insects.

However, other than the results of the present study and the high number of German SBV cases would indicate at that time, a decrease in SBV infection cases in *Culicoides* in adjacent Netherlands was shown for 2012 by Elbers et al. (2015). The percentage of biting midge pools containing SBV in the Netherlands dropped to 1.5 % (2/130, with 50 midges/pool) in 2012 but was thus still much higher than the rate of virus detection in German *Culicoides* collected in the same year (0.6 %=2/334 pools, with 3715 tested culicoids sampled between July and October, see Table 2). A similar decline became visible in Belgium, where the average SBV infection prevalence in midges had decreased from 6.7 % in 2011 to 3.6 % (35/973 pools, mean pool size=19.3 midges/pool) in 2012 (De Regge et al. 2012, 2014). Seroprevalence data of potential blood hosts confirmed the decrease of SBV circulation during 2012 in Belgium, but also revealed that the pathogen was still circulating during summer and early autumn 2012 (Méroc et al. 2013).

In Denmark, the *Culicoides* sampled in 2012 displayed a mean SBV infection prevalence of 15.8 % (41/260 pools, mean pool size=6.5 midges/pool) (Rasmussen et al. 2014) which was much higher than for other European countries in 2012 and for Denmark in the previous year (Rasmussen et al. 2012).

In 2013, all of our traps were operated on farms housing SBV-susceptible blood hosts of culicoids such as cattle, sheep and goats. The collection sites were mainly located in the southern and eastern parts of Germany (Fig. 2) where reported cases of SBV had been much lower before than in western Germany (Fig. 1). A shift of the region mostly affected by Schmallenberg disease to the east and south was therefore expected due to a relatively low herd immunity assumed among ruminants. The number of screened midges caught from July to October 2013 (n=5065, see Table 2) was much higher than that of tested culicoids collected during the same time period of the previous year (n=3715, Table 2). However, the total lack of SBV in ceratopogonids collected in 2013 suggests that the virus might have already spread across the southern and eastern parts of Germany and induced immunity in ruminants to a much higher degree than previously thought. Supporting this, interviews with livestock farmers indicated that newborns with clinical signs typical for SBV infection had frequently not been reported due to apprehended additional work and economic loss. Therefore, it seems quite possible that the actual number of SBV infections in southern and eastern Germany prior to 2013 was higher than reported, and that virus circulation in 2013 took place on a much lower level than expected.

To compare SBV infection rates in biting midges, the sampling periods of the various studies must be considered. In Denmark and France, culicoids were collected during a few days in October 2011 only (Rasmussen et al. 2012; Balenghien et al. 2014), while the sampling period lasted from August until early October 2011 in the Netherlands (Elbers et al. 2013). As a matter-of-fact, the collection periods of the studies set limits to a possible detection of SBV beforehand (Rasmussen et al. 2012; Balenghien et al. 2014; Elbers et al. 2013). Other studies sampled midges over considerably longer time periods, such as July to October 2011 (De Regge et al. 2012), May to September 2012 (Elbers et al. 2015), or May to November 2012 (De Regge et al. 2014), but only detected SBV-positive culicoids during the summer months July, August, and September (De Regge et al. 2012, 2014; Elbers et al. 2015).

Two studies investigating the occurrence of SBV in midges at various places of Poland included sampling periods from September to October 2011 and from April to October 2012. The first SBV-infected culicoids were detected only during the second year of the epidemic, namely from late August until late October 2012, following the introduction of SBV-positive bulls (Larska et al. 2013a, 2013b).

The two SBV-infected midges of our investigation were captured on August 29 and September 3, 2012, which is in accordance with SBV detection in other studies (e.g., Elbers et al. 2013; De Regge et al. 2014).

In 2014, only very few cases of SBV infections were recorded in Germany, and the disease appeared to slowly disappear from the scene. In autumn, however, several acute infections were diagnosed again in various parts of Germany, among them an open quarantine stable near Cottbus, federal state of Brandenburg, East Germany (Wernike et al. 2015). Even though the climate was relatively mild for that time of the year, the vector season was almost finished and the number of active biting midges was low, as demonstrated by the collections. None of the collected midges tested positive for SBV.

Despite the findings of several studies from northern Europe, in which SBV-positive midges could only be detected during the summer months, a transmission of SBV during the winter season cannot be excluded (Wernike et al. 2013). As there is no real biting midge-free period during the year (Mehlhorn et al. 2009), the risk of an infection might be limited during the cold season but is not absent.

Based on speculations on other arthropods possibly being involved in the transmission of SBV (Goffredo et al. 2013), it was checked whether simuliids carried the pathogen.

As none of the black flies, mainly sampled in the summer months of 2011 (Table 2), tested positive for the virus, and given the extremely low infection prevalence of biting midges, it is impossible to make a statement on a role this group of dipterans might have for the spread of SBV. Still, the involvement of hematophagous arthropods other than ceratopogonids such as biting flies or tabanids cannot be excluded at this moment.

Our findings suggest that the chances to detect SBV in the culicoid biting midge fauna are rather low outside highly epidemic periods of disease and in collections not done in close proximity to susceptible ruminants even within epidemic periods. Therefore, the establishment of a monitoring program seems useful in order to collect Ceratopogonidae at sentinel farms throughout the vector seasons and to have material available for screening in the case of a disease incident, be it Schmallenberg disease, bluetongue, or other ceratopogonid-borne diseases still to come.

The danger of a new SBV outbreak might presently be low. Over time, however, immune ruminants will be replaced by naive ones, and therefore, the declining herd immunity will increase the risk of a new epidemic (Méroc et al. 2013; Veldhuis et al. 2015).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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To investigate whether the differences in ST observations between bacteremia and urine isolates could be attributable to differences in virulence genes, VAGs of all isolates were screened by multiplex PCR. VAGs were found equally distributed across the 2 populations, with no statistically significant difference ($p = 0.675$). Comparison of serum resistance levels between urine and blood isolates also showed no phenotypic differences.

In conclusion, we found high levels of ESBL carriage and multidrug resistance in ExPEC isolates that cause bacteremia. A comparison with urine isolates provided evidence that ESBL-mediated drug resistance appears to be the selective pressure in the emergence of dominant STs in bacteremia. Future research should focus on identifying if prolonged antimicrobial drug treatment in bacteremia patients is leading to this selection.

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Transmission of Schmallenberg Virus during Winter, Germany

To the Editor: Schmallenberg virus (SBV), an orthobunyavirus, emerged in northern Europe in 2011 (*1*). SBV infection causes transient fever, diarrhea, and a reduced milk yield in adult ruminants but, most notably, stillbirths and severe malformations in lambs and calves (*2*). Insect vectors play an essential role in transmission; the viral genome has been detected in various field-collected biting midges (*Culicoides* spp.) (*3*,*4*).

During autumn 2012 and winter 2012–2013, blood samples were taken at several times from individual sheep on a farm located in the German federal state of Mecklenburg–Western Pomerania. The farm is surrounded by agricultural fields and meadows. Approximately 1,000 ewes and their lambs, a dog, and some cats were kept on the farm; most of the animals are outdoors year-round. Only dams with ≥2 lambs are housed in open stabling in December and January. The dung is regularly cleared away and stored ≈10 m from 1 of the stable entrances. Repellents or insecticides were not applied in the monitored period. Blood samples were taken in September 2012 and in January and February 2013 and analyzed by an SBV-specific real-time quantitative reverse transcription PCR (RT-qPCR) (*5*) and by an SBV antibody ELISA (ID Screen Schmallenberg virus Indirect; IDvet; Montpellier, France) by using the recommended cutoff of 50% relative optical density as compared with the positive control (sample-to-positive ratio [S/P]).

In September 2012, blood samples from 60 sheep tested negative by the SBV antibody ELISA. Moreover, fetal malformations of the brain, spinal cord, or skeletal muscle, which might have suggested a previous SBV-infection of the dam, were not

observed during the lambing season in December 2012.

On January 10, 2013, blood samples were taken from 15 sheep that had not previously been tested; samples from all animals tested negative by ELISA. However, 4 sheep (S01–S04) tested positive by RT-qPCR (quantification cycle values: S01: 31.6, S02: 39.9, S03: 37.6, and S04: 34.9). Four weeks later, antibodies against SBV could be detected. Each of the PCR-positive blood samples was injected into 2 adult type I interferon receptor-knockout mice on a C57BL/6 genetic background. Both mice that had received blood samples of sheep S01 were seropositive after 3 weeks (S/P: 207.0 and 207.2), which demonstrates the presence of infectious virus in the inoculated blood. Assuming that viral RNA remains in the blood for just a few days, as reported after experimental infection with SBV (*1*,*6*), the sheep tested in this study had most likely been infected in early 2013. During this period, the lowest temperatures rose above 5°C for several consecutive days, with a maximum of ≈9°C (Figure, panel A). Within this brief interval, when the temperature was higher, some biting midges (*Culicoides* spp.) become active (*7*). Indeed, at the end of January, a single female biting midge (Obsoletus complex) was caught in a trap equipped with ultraviolet light; the midge tested negative by the SBV-specific RT-qPCR.

On January 23 and February 20, 2013, blood samples were taken from 90 sheep that had not previously been tested (Figure 1, panel A). A viral genome was not detected in any animal at any time. However, antibodies were detectable in 9 animals on the first sampling day. In 2 additional sheep, the S/P was in the inconclusive range; 1 of the animals tested positive after 4 weeks. In the remaining 79 sheep, no SBV antibodies could be detected; after 4 weeks, 76 sheep still tested negative by ELISA. However, the S/P of 1 sheep had increased to the inconclusive range, and 2 sheep

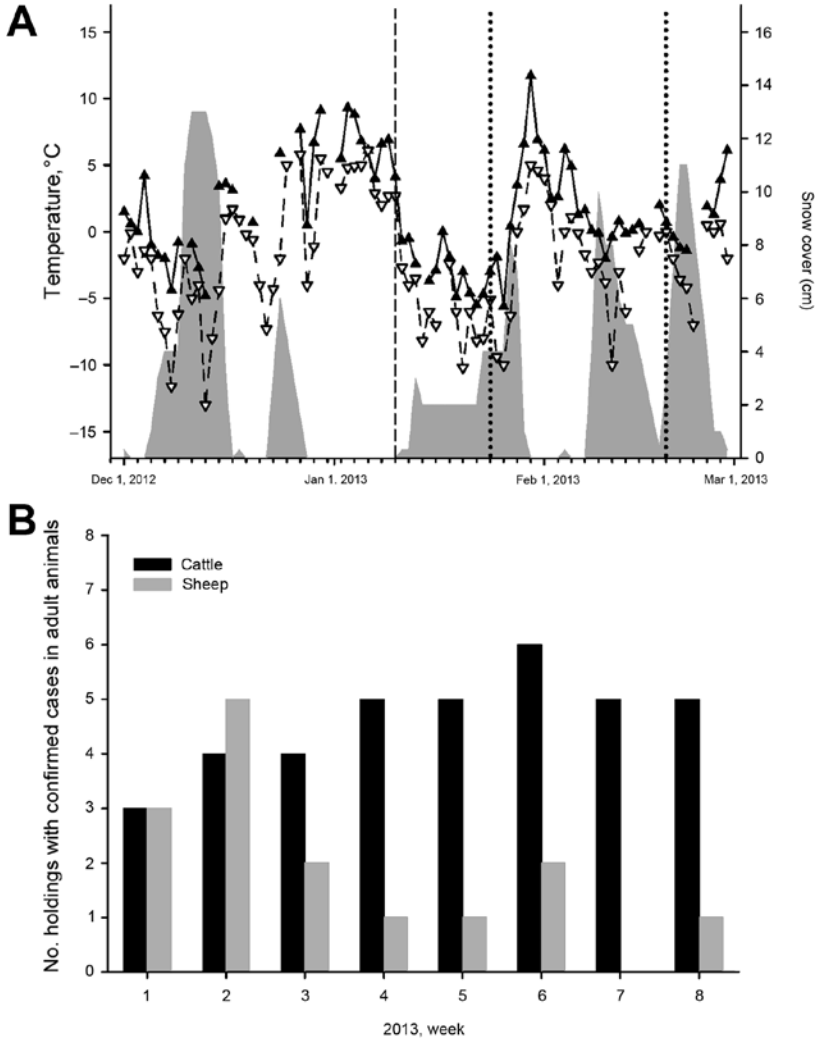

Figure. Results of analysis of samples from sheep and cattle for Schmallenberg virus (SBV), Germany, 2012–2013. A) Climate data and sampling. The maximum temperatures are shown with filled triangles and a solid line and the minimum temperatures with unfilled triangles and a broken line. Snow cover is symbolized by a gray area. The dashed line represents the day of the detection of SBV genome in 4 sheep. Further sampling days are marked by dotted lines. B) PCR-confirmed Schmallenberg virus infections in adult cattle (black bars) or sheep (gray bars) in Germany during January 1–February 20, 2013.

were seropositive. Because antibodies may be detectable 10 days–3 weeks after experimental infection for the first time (*8*), the presumed period of infection was between mid-January and mid-February. At this time, the highest temperatures again rose above 6°C for a few days (Figure 1, panel A).

Although the within-herd seroprevalence was >90% in ewes after confirmed or suspected SBV infection in 2011 (*9*), in this study, conducted during the cold season, only 12 (13%) of 90 tested sheep were positive by ELISA. Three animals seroconverted between mid-January and mid-February. Thus, SBV transmission appears to be possible at a low level, most likely because of the low activity of the involved insect vectors.

In addition to the SBV cases found on the sheep holding in Mecklenburg–Western Pomerania, an additional 52 confirmed SBV cases (defined as virus detection by qRT-PCR or isolation in cell culture) in adult ruminants were reported to the German Animal Disease Reporting System from January 1 through February 20, 2013 (Figure, panel B). Most affected animal holdings were located in Bavaria, but cases were also reported from Thuringia, Saxony, Brandenburg, Mecklenburg–Western Pomerania, Hesse, and Lower Saxony. In conclusion, transmission of SBV by hematophagous insects seems possible, even dur- ing the winter in central Europe, if minimum temperatures rise above a certain threshold for several consecutive days.

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Recurrent *Bordetella holmesii* Bacteremia and Nasal Carriage in a Patient Receiving Rituximab

To the Editor: We report a case of recurrent *Bordetella holmesii* bacteremia with 4 clinical manifestations: 3 episodes of cellulitis and 1 episode of pneumonia. The patient, a 67-year-old man, was admitted to the Pitié-Salpêtrière hospital in Paris, France, in December 2010, for recurrent cellulitis in his left leg. Eleven years earlier, diffuse large B-cell lymphoma had been diagnosed, and he had undergone 7 chemotherapy courses. He also had received 2 autologous stem cell transplants. He was receiving maintenance treatment with intravenous (IV) rituximab every 3 months and IV immunoglobulin for hypogammaglobulinemia.

The first episode of cellulitis had occurred in his left leg 2 months before admission; the condition was treated with pristinamycin (3 g/day for 14 days), and the leg healed completely. Cellulitis recurred in his left leg 2 months later; it was again treated with pristinamycin (3 g/day) for 4 days in conjunction with fusidic acid. The cutaneous lesions worsened, and

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ORIGINAL PAPER

Activity of *Culicoides* spp. (Diptera: Ceratopogonidae) inside and outside of livestock stables in late winter and spring

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Abstract *Culicoides* Latreille, 1809 midge species are the putative vectors of Bluetongue virus (BTV) and Schmallenberg virus (SBV) in Europe. To gain a better understanding of the epidemiology of the diseases, basic knowledge about the overwintering of the vectors is needed. Therefore, we investigated culicoid activity in relation to air temperature at livestock stables during late winter and spring season. Ceratopogonids were captured weekly indoors and outdoors on three cattle farms, three horse farms and one sheep farm in the federal state of Brandenburg, Germany between January and May, 2015 by BG-Sentinel UV-light suction traps. First seasonal activity was measured inside a sheep barn and cattle stables in mid-March, suggesting the existence of a preceding vector-free period. The first species at all trapping sites were members of the Obsoletus Complex followed by *Culicoides punctatus* (Meigen), 1804 and *Culicoides pulicaris* (Linnaeus), 1758 simultaneously. In total, 160 collections were made, including 3465 *Culicoides* specimens with 2790 (80.6%) of them being members of the Obsoletus Complex. The remaining 675 individuals belonged to six other culicoid species. 59.8% of all *Culicoides* were collected indoors, and almost five times as many midges were sampled on cattle farms as on horse farms. Cattle farms harboured seven species while only two species were found on the horse and the sheep farms, respectively. Temperatures, husbandry practises and the presence/quality of potential breeding sites might be responsible for the difference in species and numbers of caught specimens between livestock holdings.

Keywords Overwintering · Biting midges · Farms · Winter phenology · Vectors · Arboviruses

Introduction

Since the outbreak of bluetongue disease in Central and northern Europe in 2006, the interest attracted by culicoid biting midges (Diptera: Ceratopogonidae) as virus vectors has increased significantly. These dipteran insects are known to transmit numerous pathogens, including Bluetongue virus (BTV) (genus *Orbivirus*, family *Reoviridae*) and Schmallenberg virus (genus *Orthobunyavirus*, family *Bunyaviridae*). Both viruses are known to be transmitted by several culicoid species such as members of the Obsoletus Complex (De Regge et al. 2012; Hoffmann et al. 2009; Mehlhorn et al. 2007; Rasmussen et al. 2012) and some species of the Pulicaris Group (Hoffmann et al. 2009; Larska et al. 2013).

After their first appearance in Central and northern Europe, both bluetongue and Schmallenberg disease re-emerged in consecutive years in several European countries (Conraths et al. 2013; Hoffmann et al. 2008), infecting ruminants and causing high losses to the farming industry. The recurrence of both diseases gave reason to suggest that their pathogens were able to survive the cold winter months (Doceul et al. 2013; EFSA 2012; Hoffmann et al. 2009). Up to this day, the exact mechanisms underlying the overwintering of BTV and Schmallenberg virus (SBV) in temperate zones have remained unclear, though (EFSA 2014; Napp et al. 2011).

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The persistence of the viruses within their adult biological vectors is considered by many to be the most likely pathway of overwintering (Doceul et al. 2013; Goffredo et al. 2013; Hoffmann et al. 2008; Larska et al. 2013; Losson et al. 2007; Mehlhorn et al. 2009a, b; Tarlinton et al. 2012).

Initially, most biting midge species of the genus *Culicoides* Latreille, 1809 had been believed to outlast the winter months as larvae (Mellor et al. 2000; Rawlings and Mellor 1994; Rowley 1967). Due to the recurrence of BTV, followed by acute SBV infections during winter (Wernike et al. 2013), it was soon speculated that a constant adult midge population might remain active throughout the coldest months of the year (Hoffmann et al. 2008; Losson et al. 2007; Tarlinton et al. 2012). Some studies soon confirmed permanent midge activity during winter even in temperate regions (Hoffmann et al. 2009; Mehlhorn et al. 2009a, b). Critical voices, however, do not believe that the low adult midge population during winter is able to keep the virus circulation alive until the start of the next vector season. Therefore they suggest, that other, so far undetected pathways, might exist which can explain the persistence of viruses (Napp et al. 2011).

The present study was conducted to provide baseline information on the winter phenology of biting midges near livestock animals. We aimed to determine the culicoid species composition inside and outside of different livestock stables during late winter and spring and try to identify (species-specific) temperatures which initiate midge activity.

Materials and methods

Study period, area and sites

Insect collections took place between 25 January and 10 May 2015 at three cattle stables and three horse stables. Additionally, one stable harbouring sheep was tested between mid-March and May, 2015. All stables were located in the German federal state of Brandenburg in two regions ca. 50 km apart from each other (Table 1) and sampled on an almost weekly basis.

All stables were of solid structure and shut for the most part, but contained permanent openings useable as entrances for flying insects. They harboured exclusively cattle, horses or sheep with >5 host animals being continuously kept inside the stables. More animals of the same species were present outside at all times, belonging either to the barnyard of examination or to neighbouring farms.

Dung heaps, potential breeding sites of some Obsoletus Complex species, existed on all cattle and horse farms within a 50–100 m radius to the outdoor traps. Stables were cleaned on a daily basis, except for the sheep stable and cattle stable 1. The sheep stable was a deep-straw bedding stable, which was only cleaned after the end of the sampling period after 4 years of usage. Cattle stable 1 was a freestall and was cleaned once during the collection period in early February 2015.

Table 1 Location and number of collections at all sampling sites

Farm	Coordinates	Site	No. of samples
Region 1			
Horse 1	N 52.363679	Inside	10
	E 13.391921	Outside	10
Horse 2	N 52.375751	Inside	13
	E 13.367597	Outside	13
Horse 3	N 52.398795	Inside	10
	E 13.447769	Outside	10
Region 2			
Cattle 1	N 52.541143	Inside	14
	E 14.18044	Outside	14
Cattle 2	N 52.541157	Inside	14
	E 14.168886	Outside	14
Cattle 3	N 52.567115	Inside	11
	E 14.231117	Outside	11
Sheep	N 52.513221	Inside	8
	E 14.17472	Outside	8

Owing to only one sheep farm examined and the differing conditions between this farm and the other farms (no neighbouring farms close by, surrounded solely by fields, draughty location, no dung heaps and deep-straw bedding type), the results obtained from the sheep farm were considered additional and are therefore often discussed separately.

Biting midge collection

For the collection of biting midges, two BG-Sentinel UV-light traps (Biogents, Regensburg, Germany) were operated on every farm. One trap was installed inside each stable, the second outside, at approximately 2 m height with its trapping hole. Both traps were located in close distance to a permanent stable opening. At the same time, the inside traps were placed next to the potential hosts, the outside traps right at the stable building and also in close distance to the host animals (<30 m). All traps were run once a week for 24 h, with both traps per farm being operated simultaneously. Sampling only took place when weather conditions were suitable for midge activity (no heavy rain or wind).

A Hobo Pro v2 data logger (Onset Computer Corporation, Bourne, MA, USA) was placed right beside each indoor insect trap (at the same height within approximately 30 cm) to record the inside temperatures every 4 h during the entire sampling period. Outside temperatures were obtained from two permanent weather stations in 2 m height (region 1: Airport Berlin-

Brandenburg N 52.38, E 13.52; region 2: Müncheberg N 52.52, E 14.12).

Insects were caught and stored in ethanol (75%) until morphological identification to species or complex level under a stereomicroscope using the keys of Delécolle (1985) and Mathieu et al. (2012). Species represented by only a few individuals were further subjected to molecular analysis by COI barcoding using primers PanCuli-COX1-211F and PanCuli-COX1-727R, as described by Lehmann et al. (2012).

In the following analysis, the term ʙthreshold temperature^ marks the mean temperature of the 7 days prior to the first measured midge activity at a site or of a specific culicoid species. The mean weekly temperature instead refers to the average temperature of the named calendar week.

Results

Throughout the sampling period, 160 trap catches were made. A total of 3667 ceratopogonids were collected of which 3465 (94.5%) belonged to the genus *Culicoides*. 3372 of the culicoid midges were females (97.3%), and 80.5% ($n = 2790$) were members of the Obsoletus Complex (herein considered consisting of the species *Culicoides obsoletus* (Meigen), 1818, *Culicoides scoticus* Downes and Kettle, 1952, *Culicoides chiopterus* (Meigen), 1830 and *Culicoides dewulfi* Goetghebuer, 1936). The remaining 675 specimens represented six additional culicoid species (Table2).

More *Culicoides* specimens (59.8%) were collected inside than outside of the animal stables, equating a ratio of 1.6:1 (2072 indoors vs. 1393 outdoors). In total, the three cattle farms revealed almost five times as many sampled individuals ($n = 2860$) as the three horse farms ($n = 574$). Only 31 specimens were caught on the sheep farm (Table 2).

Species composition

The collections also show a higher number of midge species on cattle farms than on horse farms. On cattle farms, seven culicoid species could be found: the Obsoletus Complex (for this analysis regarded as one species), *Culicoides pulicaris* (Linnaeus), 1758, *Culicoides punctatus* (Meigen), 1804, *Culicoides deltus* Edwards, 1939, *Culicoides riethi* Kieffer, 1914, *Culicoides pictipennis* (Staeger), 1839 and *Culicoides poperinghensis* Goetghebuer, 1953. In comparison, only two species were collected on horse farms and the sheep farm: *C. punctatus* and Obsoletus Complex (Table 2).

Except for *C. pictipennis*, all collected culicoid species were found inside animal stables. Six species were sampled outdoors: Obsoletus Complex, *C. deltus*, *C. pictipennis*, *C. pulicaris*, *C. punctatus* and *C. riethi* (Table 2).

Table 2 Total numbers and species composition (in percentage) of sampled *Culicoides* inside and outside of each stable

Species/species group	Host stable														Total	% of total
	Cattle 1		Cattle 2		Cattle 3		Horse 1		Horse 2		Horse 3		Sheep			
	Inside	Outside	Inside	Outside	Inside	Outside	Inside	Outside	Inside	Outside	Inside	Outside	Inside	Outside		
Obsoletus Complex			235	98			155	58	9	79	136	102	25	3		80.52
Culicoides deltus																0.17
Culicoides pictipennis				1												0.03
Culicoides poperinghensis																0.06
Culicoides pulicaris	6	3		1		122										8.89
Culicoides punctatus	45	48	19	19		48	12	5		1	14	3	2	1		10.16
Culicoides riethi	1	4		1												0.17
Total	483	534	254	120	982	487	167	63	9	80	150	105	27	4	3465	100.00

First seasonal midge activity

Midge activity could be observed as early as during the 12th calendar week inside the sheep stable and 1 week later inside all three cattle stables (Table 3).

Members of the Obsoletus Complex were the first midges caught at each collection site. At some sampling sites, where midge activity started during week 16 or later (inside all horse stables and all outdoors sites), species like *C. punctatus* and *C. pulicaris* were occasionally present among the first active culicoids in addition to the Obsoletus Complex (Table 3).

The minimum and maximum temperatures recorded during the entire time of sampling (including all study sites) were −8.2 °C (weather station Airport Berlin-Brandenburg, 7 February 2015) and 26.9 °C (horse stable 2 indoors, 1 May 2015).

The threshold temperatures at the various sites were between 7.4 and 13.1 °C (Table 3) with a total mean threshold temperature of 10.5 °C for all collection sites (except sheep stable indoors due to missing data). The lowest temperatures within 7 days prior to the first sampled midge activity per site was often sub-zero, once even as low as −1.1 °C (Table 3).

The average (minimum/maximum) temperatures of the day of the first measured midge activity per collection site varied from 8.3 to 16.2 °C (Table 3), resulting in a total mean daily temperature of 11.6 °C for all trap locations.

Onset of seasonal midge activity regarding species

The first seasonally active species were midges of the Obsoletus Complex, followed by *C. pulicaris* and *C. punctatus* (Table 3) and, with some delay, by other culicoid species. This chronological order is also apparent in the species-specific threshold temperature pattern as illustrated in Table 4. Though no threshold temperature can be given for Obsoletus Complex (due to missing data), *C. punctatus* and *C. pulicaris* specimens started to show activity when the threshold temperatures rose up to 10.9 °C. Based on the early start of activity of Obsoletus Complex midges, their threshold temperature is therefore likely to be lower than 10.9 °C. Other culicoid species were collected when the threshold temperatures were much higher and reached 13.1, 15.1 and 16.9 °C (Table 4).

Except for *C. deltus* and *C. poperinghensis*, below zero temperatures were recorded within 7 days prior to the species' first activity (Table 4).

When considering only sampling days with culicoid activity, the lowest minimum temperature was −1.1 °C (week 16, horse stable 3 outdoors). The associated daily mean temperature was 8.3 °C when midges of the Obsoletus Complex were collected (Table 4).

As expected, the mean weekly temperatures recorded inside stables were higher than the corresponding temperatures outdoors. As illustrated in Fig. 1, they were highest inside the cattle stables (mean value for cattle stables 1–3), lower inside the horse stables (mean value for horse stables 1–3) and

Table 3 Time, species and temperature of the first seasonal midge activity at the various collection sites

Farm	Site	Week of first activity	Species sampled during first midge activity	Mean (lowest) temperature of 7 days prior to first activity	Mean (min/max) temperature on day of first activity
Horse 1	Inside	16	Obsoletus Complex	11.8 (7.4)	9.0 (6.1/10.8)
	Outside	17	Obsoletus Complex	10.1 (−1.1)	15.3 (7.6/23.0)
Horse 2	Inside	17	Obsoletus Complex	11.2 (4.8)	10.6 (7.6/14.2)
	Outside	17	Obsoletus Complex, *Culicoides punctatus*	9.4 (−1.1)	10.3 (1.4/18.7)
Horse 3	Inside	16	Obsoletus Complex, *C. punctatus*	12.3 (6.5)	9.3 (5.6/12.2)
	Outside	16	Obsoletus Complex	9.6 (−0.8)	8.3 (−1.1/15.2)
Cattle 1	Inside	13	Obsoletus Complex	8.4 (3.0)	12.9 (11.6/14.9)
	Outside	16	Obsoletus Complex	10.9 (−0.3)	10.4 (4.2/13.9)
Cattle 2	Inside	13	Obsoletus Complex	7.4 (2.8)	12.0 (11.1/13.6)
	Outside	16	Obsoletus Complex	10.9 (−0.3)	10.4 (4.2/13.9)
Cattle 3	Inside	13	Obsoletus Complex	9.9 (3.9)	14.9 (13.2/16.1)
	Outside	16	Obsoletus Complex, *Culicoides pulicaris*, *C. punctatus*	10.9 (−0.3)	10.4 (4.2/13.9)
Sheep	Inside	12	Obsoletus Complex	Not available	16.2 (14.1/20.4)
	Outside	19	Obsoletus Complex, *C. punctatus*	13.1 (−0.2)	12.4 (5.1/17.5)

Table 4 Time and temperature of first midge activity per species

Species	Week of first activity	Species-specific threshold temperature[a] (°C)	Minimum temperature of 7 days prior to first activity (°C)	Lowest min (mean/max) temperature during positive sampling days (°C)
Obsoletus Complex	12	No data	No data	−1.1 (8.3/15.2)
Culicoides punctatus	16	10.9	−0.3	1.4 (10.3/18.7)
Culicoides pulicaris	16	10.9	−0.3	2.6 (12.5/20.7)
Culicoides deltus	18	15.1	8.4	5.1 (12.4/17.5)
Culicoides pictipennis	19	13.1	−0.2	5.1 (12.4/17.5)
Culicoides riethi	19	13.1	−0.2	5.1 (12.4/17.5)
Culicoides poperinghensis	19	16.9	10.8	15.8 (18.7/22.0)

[a] If the first activity started simultaneously at different sites, only the lowest temperatures were considered

almost identically low outside of the cattle and horse farms (mean value for farms 1–3) (Fig. 1).

Endophily/exophily

Activity of *Culicoides* started inside cattle stables in week 13, but the number of collected specimens remained on a low level until week 16, when midges began to show activity outside cattle stables as well. An explosive increase in midge numbers was measured in week 19, the last week of sampling. This increase was mostly caused by a much higher number of Obsoletus Complex midges, though in that week, *C. punctatus* and *C. pulicaris* were caught in relevant numbers, too. All three species were more abundant inside cattle stables throughout the entire sampling period which led to a higher total indoor number of *Culicoides* (Fig. 2).

On horse farms 1–3, the first midge activity was measured in week 16 indoors and outdoors with very low specimen numbers. A temporary increase of both indoor and outdoor numbers took place in week 17 with the highest recorded number of specimens of the entire sampling period. Until week 18, more *Culicoides* were collected indoors than outdoors. Week 19 showed decreased numbers. Besides the Obsoletus Complex, which was by far the dominant species on horse farms 1–3, only *C. punctatus* was sampled in low numbers. This species appeared mostly indoors until week 18. Only in week 19 *C. punctatus* was caught in higher numbers outside the horse stables than indoors (Fig. 2).

Discussion

In the present study, we revealed a vector-free period inside and outside of stables which lasted until mid-march, 2015 (week 12). Some studies, usually conducted in countries with a milder climate (Romón et al. 2012), during milder winters (Losson et al. 2007; Mehlhorn et al. 2009a, b) or inside warm stables (Losson et al. 2007) were able to detect a constant culicoid activity throughout winter time, revealing a possible pathway of how *Culicoides*-borne arboviruses might survive the cold winter months in temperate regions. As ambient air temperatures during the time of sampling are considered to be

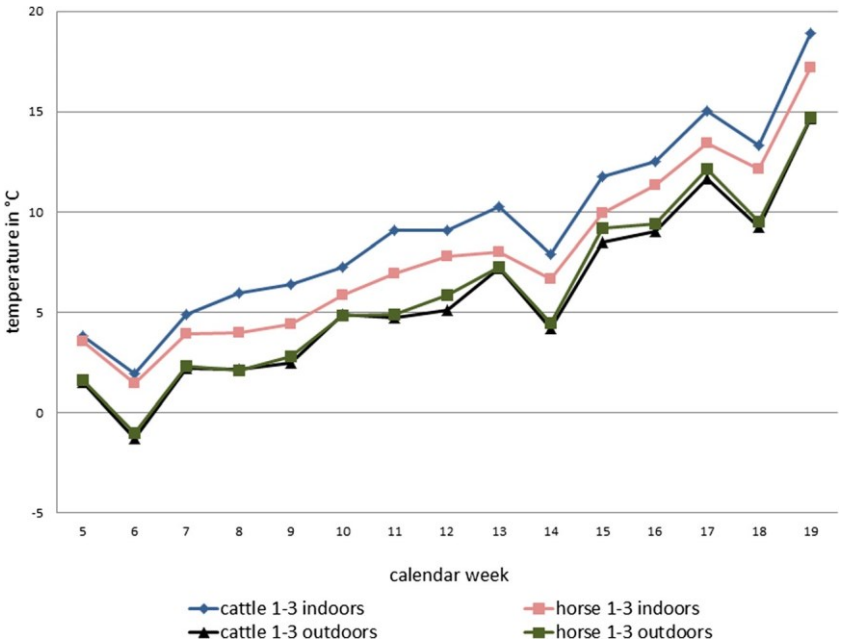

Fig. 1 Mean weekly temperatures measured inside and outside of the cattle and horse stables in calendar weeks 5–19, 2015

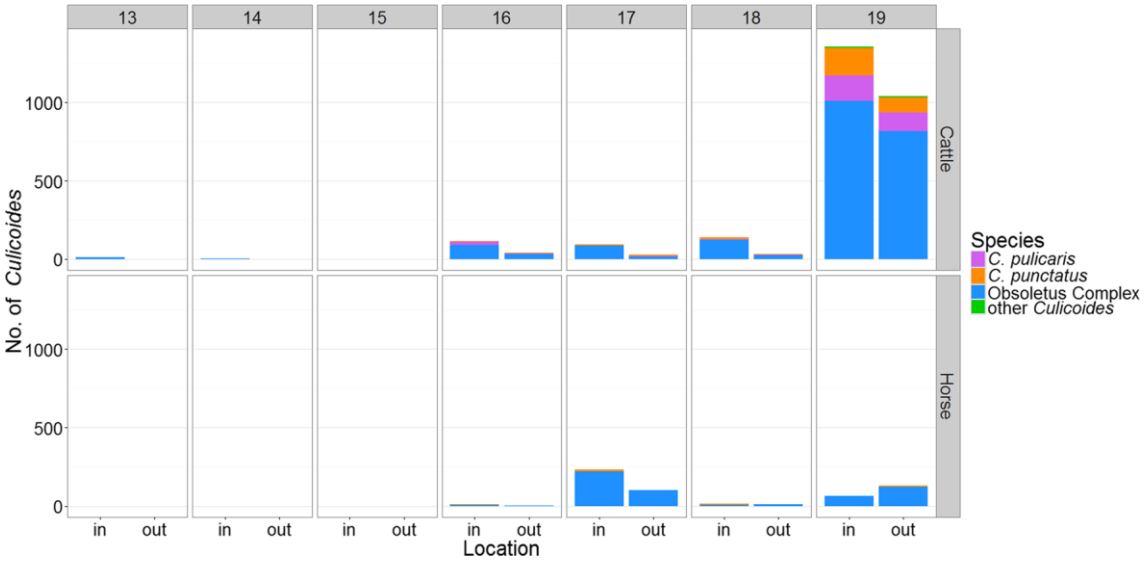

Fig. 2 Average number of caught midges in calendar weeks 13–19 inside and outside of cattle farms 1–3 (*top*) and of horse farms 1–3 (*bottom*)

the crucial factor to regulate midge activity (Lühken et al. 2015), a more detailed examination of the recorded temperatures is required when investigating the winter phenology of *Culicoides*.

First seasonal midge activity

Losson et al. (2007) showed that adult midges can remain active inside stables during winter due to a milder indoor climate. Therefore, it seemed likely that after the midge-free period in the present study, culicoid activity could be found inside stables first. Our results partly confirm the hypotheses of an early indoor activity, with midges being sampled inside the sheep and all three cattle stables. But the simultaneous start of midge activity inside the horse stables and at all outdoor sites seems to question this explanation and implies that other factors might have delayed the start of culicoid activity inside the (warmer) horse stables. We assume, that most midges at horse farms develop outdoors and, once temperatures are high enough, emerge and become active outside of the stables. Some of the active culicoids might then also enter the horse stables, leading to an almost equal number of specimens collected indoors and outdoors in week 16 in the present study (Fig. 2).

A comparison of indoor and outdoor sampling sites revealed that the mean weekly threshold temperatures were slightly above 10 °C, which is in accordance to the temperatures determined under laboratory conditions (Lühken et al. 2015).

Should our theory be correct though, that midges did not develop inside horse stables in bigger numbers, the corresponding threshold temperature for inside horse stables should not be considered. Instead, the correlated outdoor temperature of 9.7 °C (outside horse stables 1–3) seems to be the true threshold temperature for midge activity on the horse farms in the present investigation. As the lowest threshold temperature of all sampling sites was 7.4 °C (inside cattle stable 2), this field-measured temperature seems to provide sufficient warmth to initiate midge activity.

Quite often, minus temperatures were recorded within 7 days before the first midge activity took place (Table 3). Even during the day of the first measured midge activity, temperatures were as low as −1.1 °C (Table 3) which proves that even below zero degrees do not prevent midge development or adult culicoid activity, as long as the freezing is only short-termed.

Species composition

The culicoid species composition found in the present study (including all sampling sites) equates the species composition stated for most of Germany with the Obsoletus Complex being represented by 70% to >90%, the Pulicaris Complex by up to 20% of the present *Culicoides* and only relatively few specimens belonging to other culicoid species (Hörbrand and Geier 2009; Mehlhorn et al. 2009a, b). In this context, it was irrespective of the trapping sites being located indoors or outdoors. Therefore, the overall species composition seems not to be influenced by possible (temporary) endophilic/exophilic tendencies of some culicoid species in the present study.

Species composition and the number of collected midges differed strongly between livestock animals though, with cattle farms representing the most culicoid species and highest specimen numbers compared with all horse farms and the sheep farm (Table 2).

The extremely low number of sampled *Culicoides* on the sheep farm might be explained by the isolated and windy location of the farm. The differing species composition and

midge numbers between cattle and horse farms might be caused by variant husbandry practises. Better clean outs of horse stables for example, might lead to a lack of microhabitats, which might be present only in cattle stables, e.g. dried cattle dung adherent to walls as proposed by Zimmer et al. (2010). Also, the better quality of the moist cattle dung as a potential breeding substrate as compared with the drier horse and sheep droppings might add to the higher number of Obsoletus Complex specimens being caught on cattle farms as described by Thompson et al. (2013). Undetected breeding sites in the surroundings of cattle farms cannot be ruled out as well. As all study farms and their neighbourhoods had been inspected carefully before the start of the study and considered as comparable with each other, we consider this explanation as relatively unlikely, though. Based on our own experience, differences in the species composition between the two sampled regions do not exist as well.

Blood host preferences could also have had an impact on the distribution of some culicoid species between farms. Even though Obsoletus Complex midges are known to be the generalists in the genus *Culicoides* (Lassen et al. 2012), it cannot be ruled out that other culicoid species which are represented only by a few individuals and which have not sufficiently been analysed in regards to their blood feeding preferences, might just not be present on the horse or sheep farms because these livestock animals do not belong to their preferred range of blood hosts.

Onset of seasonal activity regarding species

In the present study, the species-specific threshold temperatures, triggering midge activity, differ widely (Table 4). While *C. punctatus* and *C. pulicaris* started to show activity after their threshold temperatures reached nearly 11 °C, the threshold temperatures for *C. deltus, C. pictipennis, C. riethi* and *C. poperinghensis* seem to be higher. Therefore, it may be possible, that these species may have appeared on horse farms, too, once temperatures have reached the species' threshold temperatures after the end of the sampling period. As only a low number of farms were sampled and only few midge specimens per species were collected, the species-specific threshold temperatures should be interpreted with care and provide only a first baseline dataset for future studies.Except for *C. poperinghensis* and *C. deltus*, all collected species were able to resist short-termed minus degrees, when minimum temperatures (within 7 days prior to their first activity) went down to below zero (Table 4).

Endophily/exophily

In our investigation, the total number of sampled *Culicoides* was higher indoors than outdoors with a ratio of 1.6:1, which may be attributed to the higher indoor temperatures. Previous studies proved a strong exophily for culicoid midges (Baldet et al. 2008; Baylis et al. 2010; Meiswinkel et al. 2008) and also described varying underlying conditions in their investigations, e.g. livestock movements between indoor and outdoor resting places (Meiswinkel et al. 2008) or higher outdoor temperatures caused by different sampling seasons (Baylis et al. 2010; Meiswinkel et al. 2008). Baldet et al. (2008) assumed that *Culicoides* are neither purely endophilic nor exophilic but may behave according to outdoor temperatures. The results of the present investigation show a noticeable preference for indoor locations at least during the coldest time of the year (Fig. 2). Nevertheless, the fact that more Obsoletus Complex midges were caught outside of horse stables in week 19 suggest, that their habitat preferences are not rigidly predetermined, but a reaction to environmental factors, conceivably temperature, as proposed by Baldet et al. (2008). According to our findings, the adaptation to endo−/exophilic behaviour does not only account for Obsoletus Complex midges, but also for *C. punctatus*. Though *C. punctatus* was only sampled in small numbers on horse farms, it was caught in higher numbers inside than outside horse stables except for week 19 when more specimens were collected outdoors (Fig. 2). The total numbers of collected *C. punctatus*, but also of *C. pulicaris*, reveal a marginal endophilic tendency of these species at least during the cold winter months. This is contrary to previous results published by Baldet et al. (2008) and Meiswinkel et al. (2008), who illustrated a strong exophilic behaviour for both species.

Other species like *C. riethi* and *C. deltus* were only represented by six individuals each. Due to the low number of specimens, it is impossible to evaluate, if the observed endophilic tendency for *C. deltus* and the exophilic tendency for *C. riethi* represent an actual pattern of behaviour.

Regarding endophily and exophily of the various biting midge species, it needs to be taken into account that only three horse farms, three cattle farms and one sheep farm were investigated. Thus, the data should be considered preliminary. They may illustrate the complexity of the midges' behaviour and phenology and demonstrate the necessity for more research.

Conclusion

We found a vector-free period in our investigation lasting until mid-March, 2015. We showed that the first seasonal biting midge activity was initiated by a threshold temperature of at least 7.4 °C and that even below zero temperatures did not prevent midge development or adult midge activity, as long as the freezing was only short-termed.

The type of livestock animals seemed to influence the starting time of midge activity, the species composition and the numbers of midges collected. This may be attributed to

differences in ambient temperature and, most likely, also to husbandry practises. Midges of the Obsoletus Complex were the first to become active.

Species composition hardly differs between indoor and outdoor trapping sites during early spring as most species were present both indoors and outdoors. This suggests that they are not strictly endophilic or exophilic, but that behaviour is influenced by environmental factors.

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Conflict of interest The authors declare that there is no conflict of interest.

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Field studies on breeding sites of *Culicoides* LATREILLE (Diptera: Ceratopogonidae) in agriculturally used and natural habitats

Daniela Kameke[1] ✉, Helge Kampen[2], Alexander Wacker[3] & Doreen Werner[1]

Culicoides **are vectors of pathogens mainly of veterinary importance. To establish targeted vector control measures, it is paramount to comprehend the ecological factors determining their distribution. Therefore, we used emergence traps to sample eight biotopes and assess their potential as breeding sites. Part one of the study investigates agricultural habitats, part two compares four biotopes of a forest-dominated area with less anthropogenic influence, including a physicochemical analysis of soil moisture, pH value and organic content. Thirteen culicoid species were collected, with a strong dominance of the Obsoletus Complex on meadows, and with *Culicoides punctatus* (MEIGEN), *Culicoides pictipennis* (STAEGER) and the Obsoletus Complex, to be the most abundant species in the natural habitats. Several co-existing species were found, some of them not having been described before. Our results suggest that ungrazed meadows seem unsuitable as breeding sites. Only the influence of livestock creates adequate conditions for certain midge species. The alder on fen site contained most culicoid species with the highest species diversity. Our study clearly indicates that knowledge of species-specific preferences for environmental habitat conditions (choice of breeding site) in connection to soil conditions is crucial to understand the biology and phenology of midges and their role as vectors of pathogens.**

Culicoides (Diptera: Ceratopogonidae) are known vectors of arthropod-borne viruses like African horse sickness virus (AHSV), bluetongue virus (BTV) (both genus *Orbivirus*, family Reoviridae) or Schmallenberg virus (SBV) (genus *Orthobunyavirus*, family Peribunyaviridae)[1,2]. After the outbreak of bluetongue disease in 2006 and Schmallenberg disease in 2011 in Central and northern Europe, it became apparent how little was known about the biology and ecology of the viral vectors especially with respect to *Culicoides* breeding sites and their physicochemical characteristics[3,4]. However, to be able to establish targeted vector control strategies, it is crucial to understand the biology and phenology of the species, their choice of breeding sites and the conditions potential breeding substrates must provide.

The genus *Culicoides* Latreille consists of about 96% of haematophagous species obligatorily feeding on mammals[1].

In contrast to other dipteran families like Culicidae or Simuliidae (both order Diptera), Ceratopogonidae do not contain just aquatic breeders, but also semi-aquatic and terrestrial forms. Most species of the genus *Culicoides* are semi-aquatic as larvae and breed in a wide range of habitats, showing one common feature—a relatively high level of water content[5,6]. As most culicoid species appear to be confined to one type of habitat[5], biotic and abiotic factors other than soil moisture might have an impact on the choice of breeding sites, too. Culicoid larvae feed on organic matter like fungi, algae or rotten plants[7], or are predatory on rotifers, nematodes and larvae of other invertebrates[8,9]. Therefore, the organic content of the soil and its compounds have been addressed in various studies as putative co-determining abiotic factors[3,10]. Another parameter considered as potentially influential regarding the quality of breeding substrates is the pH value of the soil[11]. Nevertheless, the overall knowledge about the specific requirements on the breeding substrate of many culicoid midge species is still very scarce.

In the present study, we examine the breeding preferences of *Culicoides* spp. by investigating eight biotopes and conducting a physicochemical analysis of four breeding substrates. The investigation consists of two parts

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Biotopes	Sampling period	No. of samples	Coordinates	Soil analysis
Region 1				
Ungrazed meadow	April–early Aug	44	N 52.761 E 14.306	
Meadow with cattle	April–late July	30	N 52.763 E 14.300	
Region 2				
Meadow with cattle	Aug–Oct	28	N 52.543 E 14.201	
Meadow with sheep	Aug–Oct	35	N 52.502 E 14.129	
Region 3				
Coniferous woodland	April–Oct	109	N 52.991 E 12.908	Moisture, pH, organic content
Deciduous woodland	April–Oct	103	N 52.991 E 12.90	Moisture, pH, organic content
Alder on fen site	April–Oct	100	N 52.991 E 12.907	Moisture, pH, organic content
Marsh area (grassland)	April–Oct	98	N 52.992 E 12.903	Moisture, pH, organic content

Table 1. Details of sampling activities during 2014 including sampling periods, sampling sites with corresponding number of valid samples and substrate analysis.

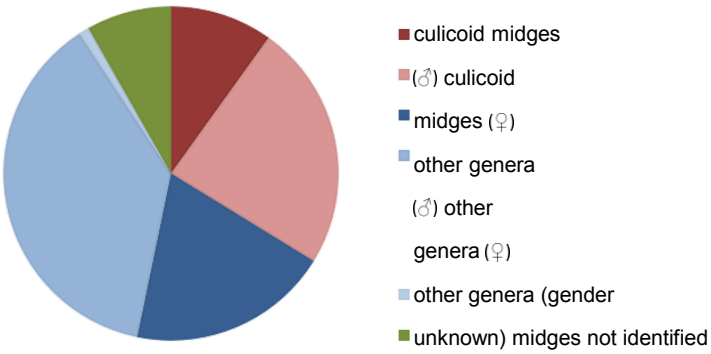


Figure 1. Composition of ceratopogonids sampled in all study sites.

to compare biotopes of high anthropogenic influence with natural habitats. Study one addresses the influence of cattle and sheep on the quality of meadows as potential breeding sites (agricultural habitats). Study two focusses on the suitability of four biotopes in a forest-dominated area and includes a physicochemical soil analysis to define the species-specific breeding conditions. The phenology of the sampled culicoid species is presented.

Results

Samples and species composition. Altogether, 547 usable samples were collected and sorted for *Culicoides*. Due to the loss or poor quality of samples, several ones had to be excluded from the study. Reasons include heavy winds or intense UV-light damaging the emergence traps, wild animals dropping the collection boxes, and people damaging or stealing traps/parts of traps.

137 samples were collected from regions 1 and 2, while region 3 was represented by 410 samples (Table 1). The total number of midges accounted for 293 individuals. While 170 specimens (110 females, 57 males; gender not determinable in 3 specimens) were species of genera other than *Culicoides* (Fig. 1), 99 belonged to 13 culicoid species (Table 2). Twenty-four midges could only be classified as Ceratopogonidae; bad condition prevented closer determination, even to genus level.

Of the 13 identified species, the Obsoletus Complex revealed the highest number with 45 specimens. Morphological identification of intact male specimens and the molecular analysis of females determined 13 of the Obsoletus Complex specimens as *C. obsoletus* s.s. and *C. chiopterus*.

The maximum number of individuals collected from the same species (*Culicoides punctatus* (Meigen), 1804) was 10 (Table 2). Most *Culicoides* (70.7%) were females, with a 2.4:1 ratio of female:male).

Species	Males	Females	Total
Obsoletus complex	12	33	45
incl. *C. chiopterus* (MEIGEN), 1830	8	0	8
incl. *C. obsoletus* s.s. (MEIGEN), 1818	2	3	5
C. achrayi Kettle and LAWSON, 1955	0	1	1
C. albicans (WINNERTZ), 1852	1	7	8
C. comosioculatus TOKUNAGA, 1956	0	1	1
C. grisescens EDWARDS, 1939	0	7	7
C. impunctatus GOETGHEBUER, 1920	2	5	7
C. kibunensis TOKUNAGA, 1937	0	3	3
C. pallidicornis KIEFFER, 1919	0	2	2
C. pictipennis (STAEGER), 1839	5	4	9
C. pulicaris (LINNAEUS), 1758	4	0	4
C. punctatus (MEIGEN), 1804	5	5	10
C. subfagineus DELÉCOLLE & ORTEGA, 1998	0	1	1
C. subfasciipennis KIEFFER, 1919	0	1	1
Total	29	70	99

Table 2. Total numbers of *Culicoides* spp. and gender composition from all study sites.

Biotope	No. of samples	No. of specimens	No. of specimens per sample	No. of species
Region 1				
Ungrazed meadow	44	0	0.00	0
Meadow with cattle	30	25	0.83	2
Region 2				
Meadow with cattle	28	6	0.21	1
Meadow with sheep	35	2	0.06	1
Region 3				
Coniferous woodland	109	8	0.07	4
Alder on fen site	100	40	0.40	10
Deciduous woodland	103	12	0.12	4
Marsh area	98	6	0.06	4
Total/mean	Σ 547	Σ 99	Ø 0.22	Ø 3.3

Table 3. Quantitative composition of culicoid biting midges per biotope.

Four Obsoletus Complex females were gravid. These comprise specimens sampled on coniferous woodland (CW) in June (1 specimen), meadow with cattle in June (2 specimens) and meadow with sheep in August (1 specimen).

Study 1: influence of cattle and sheep on meadows. Thirty-three individuals were collected on all four meadows with the highest number of *Culicoides* per sample being found on the meadow with cattle in region 1. The ungrazed meadow revealed no *Culicoides*. Later in the year, many fewer *Culicoides* per sample were collected on the meadow with cattle in region 2. The mean number of *Culicoides* collected on the meadow with sheep was even smaller (Table 3).

Mainly specimens of the Obsoletus Complex were sampled on meadows. Additionally, one individual of *C. comosioculatus* Tokunaga 1956 was collected on the meadow with cattle in region 1 (Table 4).

Study 2: biotopes in a forest‑dominated area. In total, 66 *Culicoides* were caught within the four biotopes of region 3. The alder on fen site (AFS) yielded the highest species diversity (10 species), the highest total number of *Culicoides* and the highest number of individuals per sample of region 3 (Table 3).
It also presented the highest number of specimens of a single taxon (*C. punctatus*, closely followed by the Obsoletus Complex). The remaining three biotopes contained four species each in different compositions, of which only *C. pictipennis* (Staeger) 1839 on the deciduous woodland (DW) reached a relatively high value per sample (Table 4).

Soil analysis in forest-dominated biotopes. The distribution of the measured soil factors in each biotope of region 3 are illustrated in Fig. 2a–c.

	Marsh area	Alder on fen site	Deciduous woodland	Coniferous woodland	Meadow (ungrazed)	Meadow (cattle)	Meadow (sheep)
C. achrayi	0.00	0.01	0.00	0.00	0.00	0.00	0.00
C. albicans	0.00	0.06	0.02	0.00	0.00	0.00	0.00
C. comosioculatus	0.00	0.00	0.00	0.00	0.00	0.02	0.00
C. grisescens	0.00	0.03	0.01	0.03	0.00	0.00	0.00
C. impunctatus	0.00	0.05	0.00	0.02	0.00	0.00	0.00
C. kibunensis	0.01	0.02	0.00	0.00	0.00	0.00	0.00
Obsoletus Complex	0.02	0.08	0.01	0.02	0.00	0.52	0.06
C. pallidicornis	0.02	0.00	0.00	0.00	0.00	0.00	0.00
C. pictipennis	0.00	0.01	0.08	0.00	0.00	0.00	0.00
C. pulicaris	0.00	0.04	0.00	0.00	0.00	0.00	0.00
C. punctatus	0.00	0.09	0.00	0.01	0.00	0.00	0.00
C. subfagineus	0.01	0.00	0.00	0.00	0.00	0.00	0.00
C. subfasciipennis	0.00	0.01	0.00	0.00	0.00	0.00	0.00
midges per sample and biotope	0.06	0.40	0.12	0.07	0.00	0.53	0.06

Table 4. Number of collected culicoid species per biotope and sample.

The AFS displayed the highest maximum values, but also the widest variances regarding all soil factors. It also contained the highest inter-quartile ranges for soil moisture and organic content, as does the marsh area (MA) for pH value.

Especially accounting for soil moisture, less for organic content, the inter-quartile ranges of the CW, DW and MA were on an almost equally low level. Merely the pH values of the MA showed intermediate character compared to the AFS on the one side and the CW and DW on the other side.

Statistical analysis. None of the three soil factors nor the number of collected *Culicoides* spp. was normally distributed.

The Kruskal–Wallis-test revealed that the biotopes were significantly different from each other regarding the three soil factors (with $\chi^2 = 29.86$, df = 3, $p < 0.001$ for

moisture; $\chi^2 = 44.24$, df = 3, $p < 0.001$ for pH, and $\chi^2 = 46.12$, df = 3, $p < 0.001$ for organic content). The AFS showed the highest means of each analysed soil factor (Table 5). The number of *Culicoides* differed significantly between the four biotopes of region 3, with $\chi^2 = 17.419$, df = 3, and $p = 0.001$, as revealed by the Kruskal–Wallis-test. The median test clearly illustrates that most biting midges were captured within the AFS during most sampling days (Table 6).

Logistic regression analysis showed that each soil factor had an influence on the probability of *Culicoides* to be present or not in region 3 (Omnibus test $\chi^2 = 25.95$, df = 1, $p < 0.001$ for moisture; $\chi^2 = 8.88$, df = 1, $p < 0.001$ for pH, and $\chi^2 = 14.59$, df = 1, $p < 0.001$ for organic content).

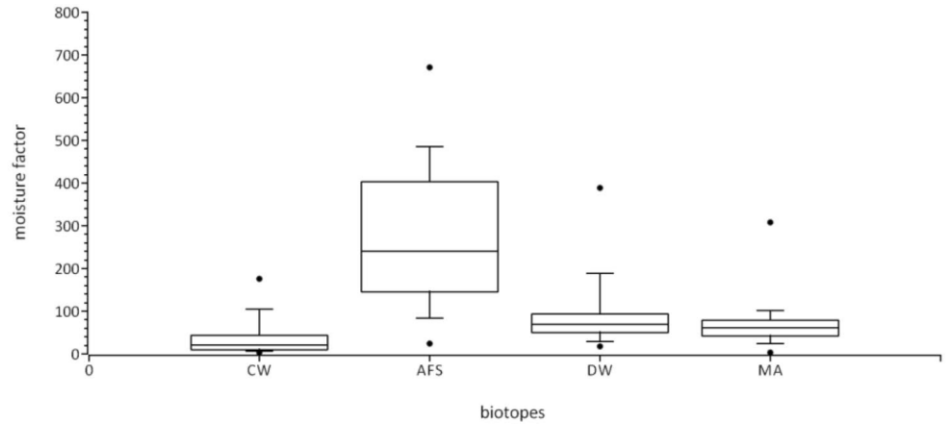
While the soil characteristics were important for presence and absence, we found no significant interrelations between the three soil factors and the number of collected *Culicoides* (linear regression model): As the bivariate correlation analysis after Pearson revealed strong correlations between the three soil factors (moisture-pH: $r = 0.686$, $p < 0.001$; moisture-organic content: $r = 0.915$, $p < 0.001$; pH-organic content: $r = 0.502$, $p < 0.001$) and weaker correlations between any soil factor and the number of sampled *Culicoides* (*Culicoides*-moisture: $r = 0.385$, $p = 0.014$; *Culicoides*-pH: $r = 0.242$, $p = 0.084$; *Culicoides*-organic content: $r = 0.274$, $p = 0.050$), an interpretation of regression coefficients is not reasonable.

Biodiversity indices. Table 7 shows the calculated diversity indices and reveals huge differences regarding the evaluated biodiversity between the three agriculturally used habitats of regions 1 and 2 and the four more natural biotopes (AFS, MA, CW, DW).

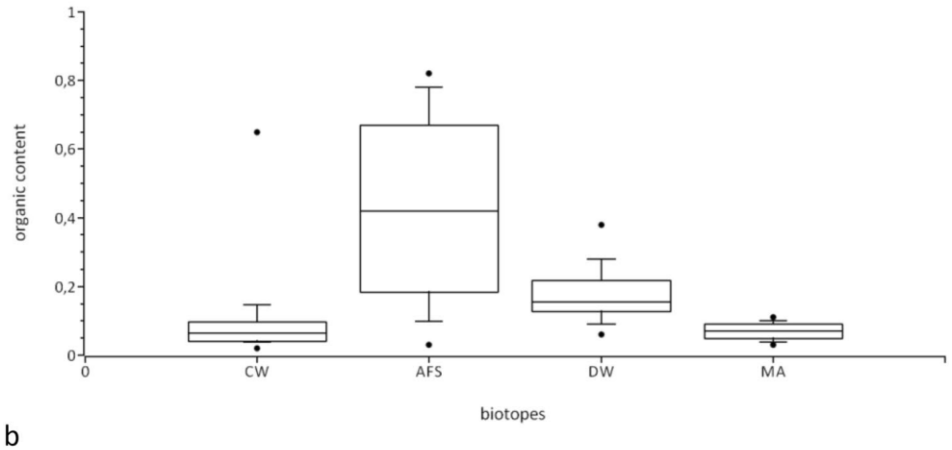
The meadows of region 2 contained only one species each, resulting in the minimum Shannon–Weaver index of zero (no diversity). The meadow with cattle of region 1 has also a very low Shannon–Weaver index (0.24) and, with two species present, an Evenness of 0.24. The Simpson index of all three meadows is either D = 1 or little less, also expressing the lack of biodiversity within these biotopes (Table 7).

Compared to the agriculturally used habitats, the four biotopes of region 3 contained more species, but with varying numbers of collected specimens. The Shannon–Weaver indices range between 1.42 (DW) and 2.96 (AFS). Population numbers of sampled *Culicoides* species were most equally balanced in the MA (0.96) and CW (0.95), closely followed by the AFS (0.89). The DW revealed a lower Evenness factor of 0.71, disclosing the dominance of one or few species in this biotope. While the Simpson index depicts the AFS and MA as the two biotopes containing the highest biodiversity of region 3 with D = 0.13, the DW reveals much less diversity reaching only D = 0.44 (Table 7).

Seasonal distribution/phenology. *Culicoides* emerged throughout the sampling period between April and October 2014. The months May and June produced most midges and the highest species diversity. The



a



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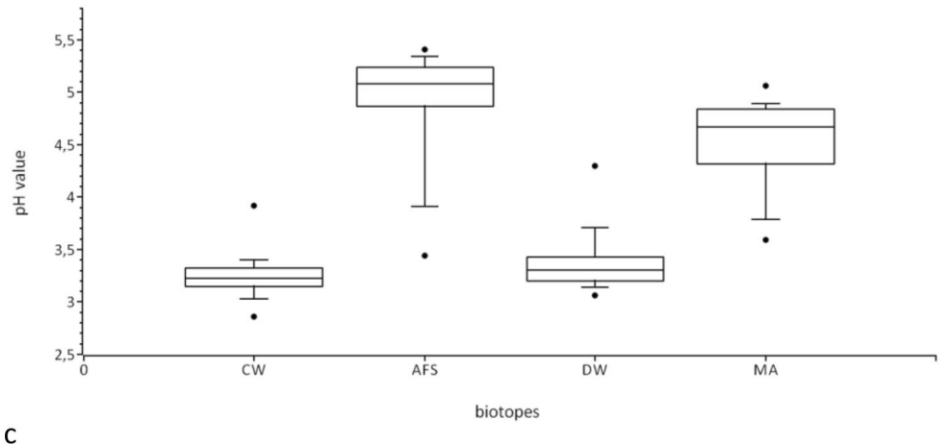

c

Figure 2. All box plots (**a**–**c**) comprise 25th and 75th percentiles (whisker box) and include the median (central line). Error bars represent 10th and 90th percentiles, with the dots delineating minimum and maximum data points. (**a**) Boxplots of the soil moisture factors measured in the four biotopes (*CW* Coniferous woodland, *AFS* Alder on fen site, *DW* Deciduous woodland, *MA* Marsh area) in region 3. (**b**) Boxplots of the organic contents measured in the four biotopes (*CW* Coniferous woodland, *AFS* Alder on fen site, *DW* Deciduous woodland, *MA* Marsh area) in region 3 (values between 0–1 correspond to 0–100%). (**c**) Boxplots of the pH values measured in the four biotopes. (*CW* Coniferous woodland, *AFS* Alder on fen site, *DW* Deciduous woodland, *MA* Marsh area) in region 3.

	Coniferous woodland	Alder on fen site	Deciduous woodland	Marsh area
Soil moisture	36.74	274.79	92.24	65.19
pH	3.27	4.79	3.38	4.53
Organic content	9	36	16	7

Table 5. Means of each soil factor per biotope.

	Coniferous woodland	Alder on fen site	Deciduous woodland	Marsh area
No. of biting midges				
> Median	3	12	4	3
≤ Median	10	1	9	10

Table 6. Results of the median test on the number of ollected *Culicoides* per biotope.

	Region 1	Region 2		Region 3			
	Meadow with cattle	Meadow with cattle	Meadow with sheep	Coniferous woodland	Deciduous woodland	Alder on fen site	Marsh area
No. of specimens (n)	25	6	2	8	12	40	6
No. of species (S)	2	1	1	4	4	10	4
Shannon–Weaver index (H)	0.24	0	0	1.91	1.42	2.96	1.92
Maximum diversity possible (H_{max})	1.00	0	0	2.00	2.00	3.32	2.00
Evenness (E)	0.24	–	–	0.95	0.71	0.89	0.96
Simpson index (D)	0.92	1.00	1.00	0.18	0.44	0.13	0.13

Table 7. Biodiversity indices of Shannon–Weaver, Evenness and Simpson index. Index numbers and calculated biodiversity indices of Shannon–Weaver index and Simpson index based on *Culicoides* spp. collected in 2014.

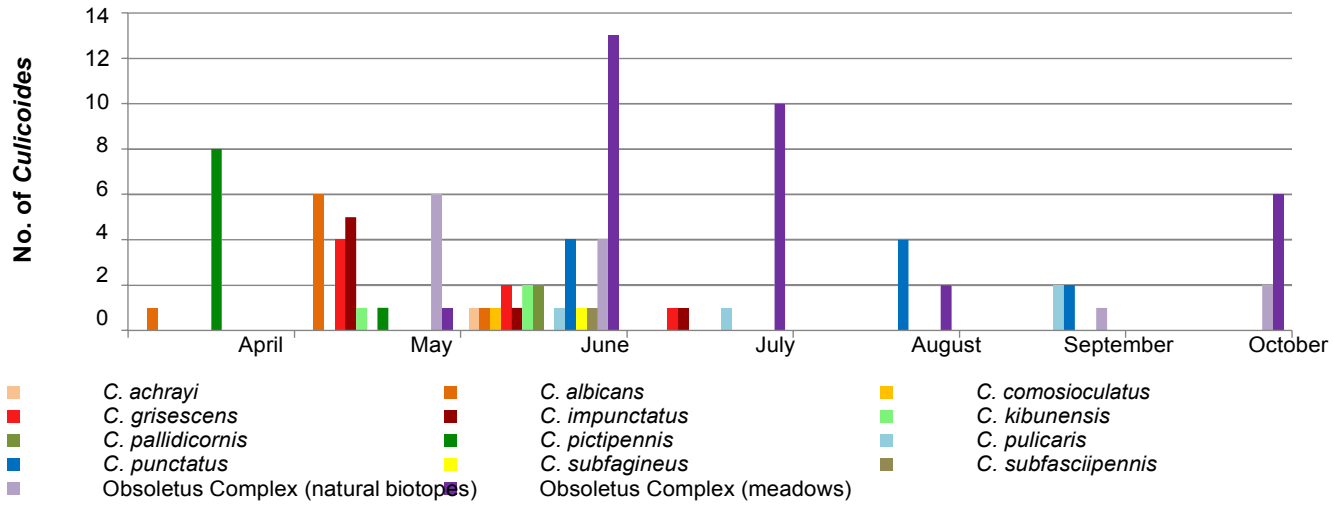

Figure 3. Phenology of *Culicoides* spp. based on all study sites sampled during 2014.

Obsoletus Complex was present for six months from May until October. Other species appeared only for a short period of time (Fig. 3).

We observed several co-habitating culicoid species, all sharing the same biotope as a developmental site (Table 8). For example, *C. albicans* occurred together with *C. pictipennis* in the DW. However, as expected, the AFS revealed most of the co-existing species with a peak in June.

Time of appearance	Coniferous woodland	Deciduous woodland	Alder on fen site	Marsh area
Mid-April			*C. pictipennis*	
Late April		*C. albicans*[a] *C. pictipennis*[a]		
Mid-May		*C. albicans* *C. pictipennis*	Obsoletus Complex *C. albicans* *C. obsoletus* s.s	
Late May	*C. grisescens* *C. impunctatus*	Obsoletus Complex	Obsoletus Complex *C. albicans* *C. grisescens* *C. impunctatus* *C. kibunensis*	Obsoletus Complex
Mid-June	Obsoletus Complex *C. grisescens* *C. punctatus*	*C. grisescens*	*C. albicans*[a] *C. kibunensis*[a]	Obsoletus Complex
			Obsoletus Complex[a] *C. impunctatus*[a]	
			C. punctatus[a] *C. subfasciipennis*[a]	
Late June			*C. achrayi*[a] *C. pulicaris*[a]	*C. kibunensis* *C. pallidicornis* *C. subfagineus*
Mid-July	*C. impunctatus*		*C. grisescens*	
Late July			*C. pulicaris*	
Mid-Aug			*C. punctatus*	
Late Aug			*C. punctatus*	
Mid-Sep			*C. pulicaris*[a] *C. punctatus*[a] *C. obsoletus* s.s	
Late Sep			*C. pulicaris* *C. punctatus*	
Mid-Oct			*C. obsoletus* s.s	

Table 8. Co-occurrence and species distribution per month and biotope of *Culicoides* spp. collected during 2014. [a] Species collected in one single sample.

Discussion

In total, 13 culicoid species were found in the present study, with 45.5% of the collected specimens belonging to the Obsoletus Complex while species only occasionally present in previous collections in Germany, accounted for approximately 25% of the sampled individuals. Thus, the species composition is only partly in accordance to earlier studies on the German *Culicoides* fauna according to which 70 to over 90% of the specimens belonged to the Obsoletus Complex and up to 20% represented members of the Pulicaris Complex, while other culicoid species were present in negligible numbers only[12,13]. However, previous studies were based on UV-light trap catches[12–15] and targeted active culicoid specimens[16]. The results obtained in this study are very specific as they

represent the species compositions associated with the respective breeding substrates.

The gender ratio differed strongly between species, revealing no pattern applicable to all species. The dominance of female *Culicoides* emerging from breeding sites corresponds to earlier results[17,18], even though the sex ratio in the present study showed a much higher proportion of females with 70.7% or a female:male ratio of 2.4:1 than the above studies with 55.6%[17] or a female:male ratio of 1.06:1[18].

The evaluation of the diversity of each biotope (excluding the ungrazed meadow where no *Culicoides* were found) revealed clear differences between the agriculturally used habitats and the more natural biotopes. The Shannon–Weaver index depicted very low diversity for all three studied meadows where biting midges were found. The two meadows (with cattle and sheep) of region 2 reached the lowest possible diversity. This seems plausible as only one species was sampled within each biotope. The meadow with cattle of region 1 revealed at least two species. The Evenness factor of 0.24 depicts the dominance of one of them. The low number of species and unbalanced number of specimens within the biotope result in a low Shannon–Weaver index of 0.24, which describes the poor level of biodiversity.

The Simpson index measures the probability that two individuals, randomly selected from a sample, belong to the same species. As only one species was sampled on each meadow from region 2, the probability to choose two specimens which belong to one species is 100% (displayed by the value of D = 1.0). The meadow with cattle of region 2 revealed at least two culicoid species, but the dominance of one species leads to a high Simpson index of 0.92 as well.

Opposite to the very low biodiversity of all meadows, the four more natural biotopes of region 3 show an overall high level of biodiversity: according to the Shannon–Weaver index, the level of biodiversity is highest within the AFS (H = 2.96). Compared to the other biotopes of region 3, the AFS revealed by far the highest numbers of culicoid species and specimens. This and the relatively high Evenness factor (E = 0.89) lead to the high H value. The Shannon–Weaver indices for CW and MA are 1.91 and 1.92, respectively. Based on the low numbers of species and specimens in both biotopes, the relatively high H value is mainly caused by its high Evenness values of 0.95 (CW) and 0.96 (MA), respectively. Therefore, the almost equal numbers of all present species leads to the relatively high biodiversity, rather than a high number of species.

The Shannon–Weaver index of the DW is the lowest of the four biotopes of region 3 with H = 1.42 and rates this biotope as the one with the lowest diversity of region 3. Though the number of species equals the one of the CW and MA, the higher number of specimens and especially the much lower Evenness factor of 0.71 reduces the H value.

Other than the Shannon–Weaver index, the Simpson index rates both, the AFS and the MA, as the two most diverse biotopes. With values of D = 0.13, the probability to randomly select two species of the same species is rather low in both biotopes. As the AFS revealed more than double as many species than the MA, the lower number of caught specimens of the MA must have led to the same biodiversity rate.

Study 1—Influence of domestic animals on meadows: up to date, dung-breeding *Culicoides* have been investigated more thoroughly[18–20] than most

other culicoid species. Most studies have focused on examining selectively either dungheaps or cowpats, rather than conducting a direct comparison between grazed and ungrazed meadows under field conditions. In the present study, we were able to show that the ungrazed meadow seems to be an unsuitable breeding habitat for *Culicoides*. Therefore, it seems plausible that the suitability of meadows as culicoid breeding sites can be largely, if not completely, attributed to the influence of livestock pasturing.

The strong dominance of Obsoletus Complex specimens sampled on grazed meadows is not surprising as this species complex is known to contain typical dung-breeders[19,20]. The high potential of manure as a breeding substrate has been demonstrated before[21,22] and explains the high quantity of *Culicoides* developing on meadows used by cattle in the present study. While 0.83 midges/sample were found on the meadow with cattle in region 1, only 0.21 midges/sample were collected on the meadow with cattle in region 2. The quantitative differences between these two study sites might be caused by the differing time periods of sampling (April to July for region 1 and August to October for region 2). Previous studies observed population peaks of Obsoletus Complex midges in October, though[23], giving reason to expect even higher numbers of midges for region 2 than for region 1, particularly so, as region 2 is an agriculturally dominated area with a higher abundance of potential blood hosts and more suitable breeding habitats than region 1.

Compared to the much higher total number of midges emerging from cowpats, sheep dung produced only two specimens. The very low number of midges originating from sheep faeces might be due to the very quick decomposition and desiccation of the rather small droppings, which likely reduces the quality of these remains as culicoid breeding sites. Therefore, it can be assumed that, contrary to pastures with cattle dung, sheep-runs might not play an essential role in promoting the distribution of *Culicoides*. For modeling approaches, it should be considered, though, that this might only apply to single scattered pieces of faeces as the longer persistence of higher volumes of sheep dung, i.e. on muckheaps, might very likely raise its quality as potential breeding sites as observed by[21].

All grazed meadows revealed very few culicoid species. Besides members of the Obsoletus Complex, only one individual of *C. comosioculatus* was found. The present investigation represents a case study though as merely one habitat of each type was sampled. More research to confirm the present results is therefore strongly recommended, even more, as ceratopogonid communities of terrestrial ecosystems have been barely investigated[24], with the consequence that breeding sites of *Culicoides* spp. are still poorly known[25].

Study 2—Quality of forest‑dominated biotopes as culicoid breeding sites: In the present study, the AFS turned out to be very productive as a culicoid breeding site in regards to the number of caught specimens and species diversity. Ten of the 13 collected species were found in the AFS. This is 2.5 times as many species as in the three other biotopes of region 3, which contained four species each in different compositions. Therefore, species- specific requirements for larval development seem to be met for more culicoid species in the AFS than in any of the other study sites.

The measured pH values are in accordance to soil analyses conducted in German forests[26]. As the top layers usually are the most acidic ones, the

chosen depth of soil sampling in the present study (upper 0–5 cm) persistently produced low pH values. Additionally, the used solvent ($CaCl_2$) is less sensitive to fast changing weather conditions, but also lowers the measured pH value significantly compared to distilled water[26]—a solvent often used in earlier studies analyzing the distribution of Ceratopogonidae.

The wide variances of the soil factors, especially moisture and organic content, were mainly caused by unequal soil conditions within each biotope rather than changes over time (unpublished data). Nevertheless, the statistical analysis revealed that all four biotopes of region 3 were significantly different from each other regarding the three soil factors. Comparing the means of each soil factor revealed that the AFS contained a higher level of soil moisture, a less acidic pH value and a higher organic content than the other three biotopes of region 3. We could show that significantly more midges (0.4 *Culicoides*/sample) developed in the AFS compared to the three other biotopes of region 3 with 0.12 (DW), 0.07 (CW) and 0.06 (MA) *Culicoides* per sample.

Previous studies have assumed that the level of moisture be a crucial factor for ceratopogonid development[17,20].
Also, some studies determined the organic content as pivotal[17,27]. Our statistical analysis revealed that each soil factor has an impact on the probability of *Culicoides* to occur. Due to high correlations between the various measured soil factors, it could not be clarified, though, whether they influence the number of specimens, too. But as many culicoid species are known to lay their eggs in batches and previous egg-laying encourages females to oviposit at the same site[28], an increase in the probability of biting midge presence should indirectly result in a higher number of specimens, too.

The aggregation of larvae in terrestrial habitats[29] typically results in a high number of samples completely devoid of midges and an overall low number of specimens sampled by emergence traps[30]. Thus, the obtained low numbers of collected specimens are not surprising. Nevertheless, emergence traps are still considered to be the best tool for the investigation of breeding site productivity, as it offers a safe assignment of species to their specific developmental sites[24,29,31].

The *Culicoides* collected in this study are discussed on species level in regards to existing literature.

Culicoides achrayi was found in the AFS. A swamp as a breeding site[32] and soil located in stagnant water[22] have previously been described for this species. We confirm June as the time of emergence[32] and add that *C. achrayi* co-exists with *C. pulicaris*.

Culicoides albicans was collected in the AFS and DW. Specimens hatched from late April to mid-June, representing one generation per year. We confirm co-habitation with *C. pictipennis* and *C. kibunensis*[11,33] and the preference for very humid substrates which has been described for the wettest parts of boglands[5,34] and for artificially waterlogged soil[11]. Our results show, that *C. albicans* larvae can tolerate medium moisture levels, too. The mean organic content of their developmental sites reached from moderate to high, and the pH values lay between strong and ultra-acidic.

Culicoides comosioculatus was found on the meadow with cattle dung in mid-June. As only one individual (a gravid female with the presumed intention to oviposit) was collected and no literature regarding breeding sites of this species could be found, our finding only indicates that this species might possibly develop in animal dung although in extremely low numbers.

Culicoides grisescens was found within the AFS, the CW and the DW from late May until mid-July. Kremer[35] listed soils of swamps and boggy grasslands as developmental sites. We collected *C. grisescens* in three different biotopes with wide variances of the mean moisture level, mean organic content and mean pH value, which reveals the wide tolerance range of this species towards these three soil factors.

Culicoides impunctatus was collected in the AFS and the CW from late May to mid-July, representing one generation per year. This finding differs from earlier observations of two generations per year in Scotland[36]. Previous studies described breeding sites as acidic, oligotrophic grasslands, swamps, boglands or marshes, often of a peaty consistence[5,10,33,34,37] and with soil pH values of 5.0–6.5 (dissolved in distilled water)[37]. This matches the pH values of the AFS in the present study (lower, but dissolved in $CaCl_2$), but excludes the much lower pH values of the CW. The range considered suitable for *C. impunctatus* larvae should therefore be extended downwards to as low as pH 2.9–3.9 ($CaCl_2$). We found *C. impunctatus* in two biotopes comprising a wide variance regarding soil moisture and organic content, which illustrates the wide tolerance range of this species. Individuals of *C. impunctatus* co-exist with Obsoletus Complex specimens as both were collected within the same sample in the AFS.

Culicoides kibunensis was collected in the AFS and MA, which matches earlier observations depicting swamps of eutrophic fresh water bodies[17,34], soil of stagnant water bodies[22] and acidic grasslands in considerable distances to swamps[33] as breeding sites. The AFS and MA revealed pH values between 3.4 and 5.4. Soil moisture and organic content displayed wide variances. All specimens hatched from late May to mid-June. *Culicoides kibunensis* was found to co-exist with *C. albicans* as observed by Kettle[33]. Earlier observations of co-habitations with *C. obsoletus* s.s. and *C. pallidicornis*[5,34] could not be confirmed.

Obsoletus Complex members were present in all study sites except for the ungrazed meadow. In the grazed meadows, Obsoletus Complex midges emerged almost throughout the entire sampling period except for the month of September. Two peaks were observed, one in June/July and a smaller one in October. As in the grazed meadows, the biotopes of region 3 also revealed two generations, but emerging at a slightly earlier time of the year with one peak in May/June and the other one in September/October.

Members of the Obsoletus Complex are known to be generalists regarding their choice of breeding sites. Only the identified member species, *C. chiopterus* and *C. obsoletus* s.s., are considered here.

Culicoides chiopterus was exclusively found on meadows grazed by cattle, which is in accordance to several earlier studies as this species is described as a dung-breeding species developing in cowpats and horse droppings[5,34,35,38].

Culicoides obsoletus s.s. was mostly sampled in the AFS. Only one individual was collected on a meadow grazed by cattle. Previous descriptions of breeding sites differed widely. Acidic grasslands in considerable distance to bogs/swamps[33]

and leaf litter compost[5,35] could not be confirmed in the present study, although the MA and AFS were of a comparable character. While Uslu and Dik[17] could not find any *C. obsoletus* s.s. in wet organic matter-rich soil, we collected most specimens of this species in the AFS and can therefore confirm previous findings[11,29,32,39]. The time of *C. obsoletus* s.s. activity in Germany (April–October) as described by Havelka[32] agrees with our observations.

Culicoides pallidicornis was found in the MA in late June. This species revealed the smallest variances of all sampled biting midge species regarding the three soil factors, using soil with pH values of 3.6–5.0 ($CaCl_2$) and a relatively low level of moisture. This contradicts earlier observations where *C. pallidicornis* developed in the mud of eutrophic fresh-water swamps[5]. While *C. pallidicornis* larvae are known to co-exist with *C. kibunensis*[5], we can add *C. subfagineus* to share the same developmental site.

Culicoides pictipennis was collected in the DW and, to a minor part, in the AFS. The preferred physicochemical breeding conditions were ultra to extremely acidic with a medium moisture level and a moderate to slightly increased organic content. This differs from previous studies, which have found this species to develop only at the margin of stillwater bodies like pools and ponds, and the littoral of lakes or in artificially waterlogged soil[11,32,34]. Havelka[32] observed *C. pictipennis* between May and June, while in our investigation the first specimen emerged as early as mid-April. We can confirm the co-existence of *C. pictipennis* and *C. albicans* as previously observed by Harrup[11].

Culicoides pulicaris was sampled in the AFS from late June until September, which agrees with observations denoting May to September as the activity time of this species[32]. *Culicoides pulicaris* seems to prefer breeding substrates with a high moisture level and a high organic content, as previously described[17,32,34]. We can add that *C. pulicaris* breeds in soil showing pH values at least between 4.0 and 5.4. We collected *C. pulicaris* together with *C. achrayi* and found it to simultaneously emerge from one biotope with *C. obsoletus* s.s. Additionally, we can confirm the co-existence of *C. pulicaris* with *C. punctatus*[5,40], since both species have similar breeding habitat preferences[11].

Culicoides punctatus was sampled in the AFS and, to a minor part, in the CW. Time of emergence was from mid-June to late September, which is in accordance with earlier observations listing April-August and October as times of activity[32]. In the present study, a strong preference for swampy conditions with soil of high moisture, high organic content and a strong to very strong acidity was found. This is in agreement to previous findings[11,32,41]. The co-existence of *C. punctatus* with *C. pulicaris* is well known[5,40] and can be confirmed once more. Additionally, we found *C. punctatus* to co-occur with *C. subfasciipennis*.

Culicoides subfagineus was caught in the MA in late June. The soil was oligotrophic and contained a relatively low moisture level with pH values between 3.6 and 5.0. The first record of this species in Germany was in 2014, when *C. subfagineus* was observed to attack cattle[42].

Culicoides subfasciipennis was sampled in mid-June in the AFS. The time and choice of breeding site are in accordance to previous findings[17,32]. Breeding conditions for the only individual collected revealed a medium soil moisture factor, a pH value of 5.2 and a medium organic content. The species was found to co-develop with *C. punctatus*.

Conclusion

By conducting a direct on-the-field comparison, we were able to show that ungrazed pastures seem to be unsuitable breeding habitats for biting midges and that solely the use of pastures by domestic animals create appropriate breeding conditions for few culicoid species. While pastures with the influence of cattle produced the highest numbers of specimens, mainly of Obsoletus Complex midges, the influence of sheep was far less productive.

All four "natural" biotopes of study 2, situated in a forest-dominated area, produced less specimens per sample than the pasture with cattle, but a higher species diversity. Most individuals and the highest diversity of culicoid species were found in the AFS, which contained the highest means of organic content, soil moisture and pH value. In each of the biotopes 'CW', 'DW' and 'MA', four biting midge species were collected, but with different species compositions. The number of emerging specimens and species was highest in May and June, with some species being limited to a short time period of appearance (e.g. *C. achrayi*) while other species developed almost throughout the season (e.g. Obsoletus Complex, *C. punctatus*). The 13 collected species differed widely in their choice of breeding sites and therefore also in their breeding substrate preferences. Every single one of the measured soil factors (moisture, organic content, pH value) has a statistical influence on the probability of culicoid midges to occur. To understand the biology and phenology of biting midges and their role as vectors of pathogens, it is of high importance to gain closer knowledge of species-specific preferences for breeding sites in combination with physicochemical properties and the agricultural pasture use.

Methods

Insect collection and identification. Insect collection took place during the summer 2014 at several sites in three different regions of the federal state of Brandenburg, Germany (Table 1).

Ten emergence traps (ground area 30 × 30 cm^2) were positioned randomly within each studied biotope. Collection took place every two weeks, with the traps afterwards being moved to new sites within the biotope. Occasionally the traps captured gravid midges, which must have foraged on the sampled substrate before, or entered the enclosed space during the process of placing the traps, with the intention to oviposit. Therefore, gravid midges were not excluded from the analysis but considered as a representation of the substrate's potential as a suitable breeding site. Biting midges were collected and stored in ethanol (75%) until further analysis. Identification to species or complex level was conducted under a stereo microscope following the identification keys of Delécolle[43] and Mathieu et al.[44]. Specimens of poor condition, which could not be identified morphologically, were subjected to molecular analysis by COI barcoding using primers PanCuli-COX1-211F and PanCuli-COX1-727R[45].

Some of the specimens belonging to the species *Culicoides obsoletus* (Meigen) 1818, *Culicoides scoticus* Downes and Kettle 1952, and *Culicoides chiopterus* (Meigen) 1830, plus the isomorphic species *Culicoides dewulfi* Goetghebuer 1936, which could neither be determined by a morphological nor by molecular approach, are referred to as Obsoletus Complex.

Study sites. *Study 1: influence of cattle and sheep on meadows.* To determine the influence of cattle and sheep on the quality of meadows as potential breeding habitats of culicoid midges, an ungrazed meadow, meadows with cattle dung and a meadow with sheep dung were investigated in two different regions, for reasons described below. The comparison of an ungrazed meadow with a meadow with cattle dung was conducted in region 1, which was located in the far eastern part of the federal state of Brandenburg, Germany, close to the Oder River on the Polish border and had occasionally been flooded in previous years. The ungrazed meadow had not been used as a pasture for more than ten years. The comparison of the influence of cattle vs. sheep on meadows was conducted in region 2, an agriculturally dominated area, located in the nature reserve Märkische Schweiz, Brandenburg, Germany (Table 1).

Due to the dry summer 2014, the farmer had to use the ungrazed meadow for his livestock animals, which lead to the loss of the ungrazed meadow for this project. A short-termed change of plans was inavoidable and resulted in the second comparison between a meadow with cattle and a meadow with sheep in region 2.

Study 2: biotopes of a forest‑dominated area. Following the approach described above, insects were sampled in four biotopes of region 3: coniferous woodland (CW), deciduous woodland (DW), alder on fen site (AFS) and marsh area (MA) (Table 1). The area is a forest-dominated territory, positioned in northwestern Brandenburg, Germany, presenting wide patches of deciduous and coniferous woodland. The emergence traps of the AFS were placed along the littoral zone within the muddy area.

Soil sampling and analysis. In region 3, soil samples were taken to analyse soil parameters such as water content (moisture factor), acidity/alkalinity (pH value) and organic content.

Except for days with heavy rainfall (April and end of June), each soil sample was taken at the beginning of the fortnightly collection period right beside the emergence traps and obtained from the upper layers of substrate (0–5 cm) using a hand shovel. Samples were immediately processed after arrival in the laboratory.

Soil moisture factor. Each soil sample was freshly weighed (fresh weight), dried at 105 °C for approximately 24 h to obtain a constant weight and then re-measured (dry weight). The moisture factor was calculated as follows:

$$\text{Moisture factor} = ((\text{fresh weight} - \text{dry weight}) / \text{dry weight}) * 100$$

pH value and organic content. On 12 June, 23 July and 17 September 2014, ten soil samples per biotope were taken as described above. In the laboratory, each sample was immediately dried at 40 °C. After oven-drying, all samples were passed through a 2 mm sieve.

pH-values were measured with a WTW Multimeter 3410 (Weilheim, Germany) using a 0.01 M $CaCl_2$ solution (5 ml soil + 25 ml $CaCl_2$ solution), following the standard procedure of the HFA 3.1.1.7[46].

The classification for soil pH values of the United States Department of Agriculture Natural Resources Conservation Service was used[47].

The remaining sieved soil samples were dried for 24 h at 105 °C to a constant weight and then used for evaluating the organic content following the LOI (loss-on-ignition) method (5 g per sample ashed for 4 h at 550 °C). The organic content corresponds to the mass leaking as gas during the ignition process and is related to the dry weight:

$$\text{Loss on ignition} = (\text{dry weight} - \text{ashed weight}) * 100 / \text{dry weight}$$

The three parameters 'soil moisture', 'pH value' and 'organic content' were subjected to statistical analysis.

The statistical analysis was conducted using the programme SPSS (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY).

Due to the high number of zero-values regarding the presence of biting midges, the analyses were based on the bi-weekly sum of *Culicoides* numbers and the means of each soil factor per biotope.

While the soil moisture factor represents a fast changing environmental variable, the pH value and the organic content are known to be relatively constant factors, which usually only vary over a longer time period. Therefore, the only slowly varying pH value and organic content obtained for the three sampling dates 12 June, 23 July and 17 September 2014 were regarded representative for the collection sites throughout the season (interpolation of data).

The three soil factors and the number of *Culicoides* spp. were individually tested for normal distribution utilizing the Kolmogorov–Smirnov-test. For a better understanding of the constitutions of the biotopes, the Kruskal–Wallis-test (as a non-parametric test for not-normally distributed data) was used, and the means of each soil factor for each biotope was calculated. The test was also chosen to check for differences regarding the number of collected *Culicoides* spp. between the four biotopes, followed by a median-test.

The logistic regression model was used to assess the influence of each soil factor on the probability of *Culicoides* spp. to occur. As strong correlations between the soil factors were measured, the regression for each soil factor was calculated separately. A bivariate correlation analysis after Pearson was conducted to examine whether the number of collected *Culicoides* spp. correlated with any of the soil factors.

Biodiversity indices. To assess the diversity of the studied biotopes and the examined midge fauna, the Shannon–Weaver index (H), the Evenness (E) and the Simpson index (D) were calculated. All biotopes were subject to diversity analysis, except for the "ungrazed meadow", as no *Culicoides* were detected.

The Shannon–Weaver index was calculated as follows:

$$H = -\sum p_i * \log_2 p_i$$

with p_i = the proportion of the total sample epresented by species i and $p_i = n_i / N$.

For further calculation, the maximum diversity possible (H_{max}) was established:

$$H_{max} = -\log_2 S$$

with S = number of detected species (species richness), so that the Evenness (E) could be calculated:

$$E = H / H_{max}$$

The Simpson index, measuring the probability of two individuals randomly selected from one sample to belong to the same species, was calculated as follows:

$$D = \sum n (n-1) / N (N-1)$$

N = number of species.

Ethics declaration:research involving human and animal participants. This study did not involve protected animal species and no human participants were involved in the work.

Infomed consent. All authors consent to submission of this manuscript.

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Author contributions

All named authors declare that they have made substantial contributions to the presented work and have given approval of the submitted version. D.K. was involved in all aspects of the presented work, A.W., D.W. and H.K. were involved in drafting and revision of the manusript. H.K. was responsible for the conduction of the molecular identification of ceratopogonids.

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3. Summary

Midges are small mosquitoes that can transmit pathogens to susceptible hosts through their blood-sucking act. They are known as biological vectors that can transmit the bluetongue virus (BTV) and the Schmallenberg virus (SBV) to ruminants, among others. Various vector control measures can be used to curtail the spread of the virus during an epidemic. However, for effective vector measures, it is essential to have profound knowledge of the role of biting midges as vectors, as well as their biology and phenology. For several years, midges were not in the focus of research and there are still considerable gaps in knowledge. Therefore, the present work examines various aspects of biting midges of the genus *Culicoides*, whose function as vectors of the Schmallenberg virus was already proven at the beginning of the project.

The aim of the first part of this work was to determine the percentage of infected midges in various German areas in order to determine the influence of *Culicoides* midges within the virus epidemic. For this purpose, samples, collected during 2011 and 2012 as part of monitoring projects, were analysed. Additionally, in early 2013, various farms in southern and eastern regions of Germany, where SBV was considered to be largely absent, were equipped with UV traps. The small number of virus-positive samples did not allow a more precise assessment of the viral spread in culicoid midges. Instead, it revealed the importance to conduct targeted samplings of its vectors during an acute outbreak. Additionally, the presented results and statements made by several animal owners, gave reason to believe, that SBV must have affected the southern and eastern parts of Germany earlier than actually assumed. This would consequently have led to an increased immunity in host animals, which provides a reasonable explanation for the low positive values and is in agrement with the statements made by various farmers.

The second part of this work identifies the conditions and surrounding factors under which acute SBV diseases emerged in ruminants in the cold winter months of 2012/2013. After the diagnosis of several acute SBV infections of sheep in a sheep pen in Mecklenburg-Western Pomerania, culicoid midge activity could be proven. This demonstrates that, suitable conditions for its vectors given, an infection of SBV can also take place during wintertime. A more detailed analysis of the surrounding conditions revealed, that the outdoor temperatures during infection were consistently at values of at least 5-9 ° C for several consecutive days, which enabled the flight and blood-sucking activity of the midges within

the shelter.

Midge activity during wintertime represents a crucial component in understanding how the virus can outlast the cold season. A constant midge presence could lead to a low but permanent infection rate throughout the cold months, enabling a recurrence of the pathogen the following year. Instead, a longer vector-free timeperiod would point to other mechanisms that allow the virus to re-occur in Germany on a yearly basis. Thus, the acute cases of SBV infections in sheep rose the question of critical threshold temperatures, representing the beginning of midge activity. The investigation of several stables sheltering cattle, horse or sheep addressed potential differences between indoor and outdoor activity and whether the type of host animal has an influence on the beginning of the flight. In the third part of this work, a long vector-free period and several differences in the onset of midge activity between different types of host animals could be detected. It could also be illustrated that the progression of the flight began differently depending on the present type of host animal/type of stable. For all cattle stables and the sheep barn the first midge activity was measured indoors, whereas for horses, culicoid midges were found to become active either at the same time or almost simultaneously inside and outside the animal shelters. This suggests that the horse stables do not represent good breeding sites for midges, which might be attributed to husbandry practices. In addition, it was possible to determine specific threshold temperatures for the different types of host animals and for various midge species. Altogether, the late beginning of flight, measured at the beginning of March, was surprising. This raises more questions of alternative mechanisms enabling the virus to outlast the winter months. The documentation of species-specific threshold temperatures can be a useful tool f.i. within automated large stables to keep indoor temperatures under the threshold value in order to postpone the onset of culicoid activity of various vector species. This may help to prevent virus transmissions during winter or to evoke a delay in spring, making it more difficult for the virus to overwinter.

To be able to start instant defense measures during an ongoing virus epidemic, which is transmitted by *Culicoides* midges, reducing the ground-living midge larvae offers a promising option during the warm season. For targeted vector control measures, it is important to know the breeding sites of culicoid midge species. Therefore, four agriculturally used biotopes were sampled and compared to four biotopes of a forest-dominated area. The results clearly show that meadows per se are not suitable breeding habitats for *Culicoides*

spp. Only the influence of livestock animals induces their potential as developmental sites. The various biotopes of the forest-dominated region were less subject to anthropogenic influences. Although fewer individual midges were found here, it displayed a higher biodiversity than the agricultural habitats. These results demonstrate once more the potential of forests in regards to the preservation of biodiversity. In Particular, the alder on fen site revealed most midge species and also the highest number of collected specimens among the studied biotopes. That illustrates the high impact of this specific humid type of habitat in respect to species diversity and the need of its perpetuation.

As part of this work, new breeding sites for a variety of culicoid species were identified and assigned to the usually rather short profiles of known *Culicoides* species. For one part, previous observations of chosen substrates could be consolidated. Furthermore, new breeding substrates were identified. Additionally, information of abiotic factors such as ph-value, soil moisture or organic compound of all sampled breeding substrates obtained from a soil analysis, extended the knowledge about the species-specific choice of breeding habitats and their characteristical traits. The additional knowledge about potential breeding substrates and their soil factors might be useful for future epidemiological modelling approaches. It can also raise the effectiveness and accuracy of targeted vector control measurements during an epidemic outbreak. Therefore, it may indirectly contribute to the preservation of endangered rare species. However, there is still an enormous need for more research before this goal can be fully achieved.

4. Zusammenfassung

Gnitzen sind kleine Mücken, die durch ihren Blutsaugeakt Krankheitserreger auf empfängliche Wirte übertragen können. Sie sind als biologische Vektoren bekannt, die u.a. das Blauzungen Virus (BTV) und das Schmallenberg Virus (SBV) auf Wiederkäuer übertragen können. Um während einer Epidemie die Virusverbreitung einzudämmen, können verschiedene Maßnahmen im Rahmen der Vektorkontrolle eingesetzt werden. Für eine effektive Bekämpfung der Überträger ist es jedoch unbedingt erforderlich, eingehende Kenntnisse über die Rolle der Gnitzen als Vektoren sowie ihre Biologie und Phänologie zu besitzen. Gnitzen standen jedoch lange Zeit nicht im Fokus der Forschung und nach wie vor gibt es erhebliche Wissenslücken. Die vorliegende Arbeit untersucht daher verschiedene Aspekte von Gnitzen der Gattung *Culicoides*, deren Funktion als Überträger des Schmallenberg Virus bereits zu Beginn des Projektes als bewiesen galt.

Der erste Teil dieser Arbeit hatte zum Ziel, den prozentualen Anteil an infizierten Gnitzen in einigen Gebieten Deutschlands zu ermitteln, um die Bedeutung der Mücken am Virusgeschehen zu erfassen. Dazu wurden Proben analysiert, die 2011 und 2012 im Rahmen von Monitoringprojekten gesammelt worden waren. Zudem wurden im Frühjahr 2013 diverse Betriebe in südlichen und östlichen Regionen Deutschlands, die bis dahin noch als relative freie SBV-Zone galten, mit UV-Fallen ausgestattet. Die geringe Anzahl an viruspositiven Proben ließen eine genaue Einschätzung der Viruslast in Gnitzen zwar nicht zu, zeigte aber stattdessen, wie wichtig eine gezielte, frühzeitige Probenahme der Vektoren während eines akuten Ausbruchs ist. Zudem lieferten die Ergebnisse und Erfahrungen insbesondere durch Aussagen der Tierwirte wichtige Hinweise darauf, dass auch die südlichen und östlichen Teile Deutschlands schon früher als vermutet mit dem Schmallenberg Virus durchseucht worden sein müssen. Dies hätte folglich eine erhöhte Immunität der Wirtstiere hinterlassen, was eine schlüssige Erklärung für die geringen Positivwerte liefert und mit den Aussagen diverser Landwirte übereinstimmt.

Im zweiten Teilprojekt konnte gezeigt werden, unter welchen Bedingungen es in den kalten Wintermonaten des Jahres 2012/2013 zu SBV-Erkrankungen von Widerkäuern kam. Nach dem Auftreten einiger akuter SBV-Infektionen mehrerer Schafe in einem Schafstall in Mecklenburg-Vorpommern, konnte die Präsenz und Flugaktivität von Gnitzen nachgewiesen werden. Dies zeigt, dass, sofern für die Vektoren passende Bedingungen herrschen, Übertragungen von SBV auch im Winter durchaus möglich sind. Die in eingehenderen

Analysen ermittelten Temperaturen lagen für den Zeitraum der Übertragung für einige Tage durchgehend bei Werten von mindestens 5-9 °C, was die Flug- und Blutsaugaktivität der Gnitzen ermöglichte.

Die Gnitzenaktivität im Winter ist ein wichtiger Baustein im Verständnis darüber, wie das Virus die kalten Monate überdauern kann. Eine durchgängige Präsenz der blutsaugenden Insekten könnte das Infektionsgeschehen auf niedrigem Niveau über den Winter hinweg aufrecht erhalten und für ein erneutes Aufflammen in der kommenden Saison führen. Eine längere vektorfreie Zeit würde jedoch auf andere Mechanismen hindeuten, die es dem Virus ermöglichen, jährlich erneut in Deutschland aufzutreten. Somit ergab sich aus dem akuten Infektionsgeschehen an Schafen die Frage nach den kritischen Grenzwert-Temperaturen, ab denen mit einer Gnitzenaktivität zu rechnen ist. Es wurde untersucht, ob sich die Aktivität vorerst nur auf die wärmeren Innenbereiche der Tierstallungen beschränkt und ob die Art des Wirtstieres einen Einfluss auf den Flugbeginn nimmt.

Im dritten Teil konnten eine längere vektorfreie Zeit und diverse Unterschiede bezüglich der beginnenden Gnitzenaktivität zwischen verschiedenen Wirtstieren festgestellt werden. Auch konnte gezeigt werden, dass der Flugverlauf je nach Wirtstier/Stalltyp verschieden begann: sowohl bei Rinderställen wie auch beim Schafstall erfolgte die erste Gnitzenaktivität innerhalb der Stallungen, bei Pferden entweder zeitgleich oder zumindest zeitnah innerhalb wie auch außerhalb der Behausung. Dies deutet darauf hin, dass Pferdeställe keine guten Brutstätten für Gnitzen darstellen, was auf die Art der Säuberung zurückgeführt werden könnte. Zudem konnten sowohl für die unterschiedlichen Wirtstierställe als auch für die verschiedenen Gnitzenarten spezifische Grenzwerte ermittelt werden, ab denen mit einer Aktivität gerechnet werden muss. Insgesamt überraschte der recht späte Flugbeginn Anfang März, was die Frage nach alternativen Überwinterungsmechanismen weiter öffnet. Die Erfassung artspezifischer Aktivitätsgrenzwerte kann u.a. dafür genutzt werden, um die Innenbereiche von automatisierten Großställen unter dem kritischen Temperaturwert zu halten und so eine Verzögerung des Aktivitätsbeginns entsprechender Vektorarten herbeizuführen. Dies könnte dazu beitragen Virusübertragungen im Winter zu verhindern oder im Frühjahr zumindest für einige weitere Wochen hinauszuzögern, um dem Virus die Überwinterung zu erschweren.

Um im Zuge einer Virusepidemie zügig reagieren zu können, bietet die Bekämpfung der

bodenlebenden Gnitzenlarven während der Vegetationsperiode eine gute Möglichkeit. Für gezielte Bekämpfungsmaßnahmen ist es jedoch wichtig die potentiellen Bruthabitate von Gnitzen zu kennen. Daher wurden vier Biotope im Agrarbereich beprobt und vier Biotopen einer forstdominierten Region gegenübergestellt. Die Ergebnisse zeigen sehr deutlich, dass Weideflächen a nicht als Bruthabitat für *Culicoides*-Arten geeignet sind. Lediglich der Einfluss durch die Wirtstiere bewirkt ihr Potential als Entwicklungsort. Die unterschiedlichen Biotope der forstdominierten Region unterlagen weniger anthropogenen Einflüssen. Hier wurden zwar insgesamt weniger Gnitzen gefunden, dafür jedoch eine höhere Artenvielfalt als in den beweideten Flächen. Dieses Ergebnis zeigt einmal mehr die große Bedeutung des Waldes zum Erhalt der Biodiversität auf. Unter den forstdominierten Biotopen wies der Erlenbruch die meisten Gnitzen sowie die höchste Artenvielfalt auf und stellt neben seinem Beitrag zur Artenvielfalt auch die Wichtigkeit seines Erhaltes dar.

Im Rahmen dieser Arbeit konnten zudem neue Bruthabitate ausfindig gemacht und den meist sehr kurzen Profilen entsprechender Gnitzenarten zugeordnet werden. Zum Teil konnten die Funde frühere Ergebnisse festigen oder mittels der durchgeführten Bodenanalyse die Erkenntnisse über Ansprüche, die Gnitzen an „ihr“ Brustsubstrat stellen, erweitern. Die neuen Informationen können für zukünftige epidemiologische Ausbreitungsmodelle genutzt werden und dazu beitragen, dass Vektorarten im Zuge einer Bekämpfung zielgerichteter bekämpft werden können ohne dabei seltene Arten zu gefährden. Nach wie vor ist bis zum Erreichen dieses Ziels jedoch noch ein enormer Forschungsbedarf gegeben.

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