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Breeding sites and species association of the main Bluetongue and Schmallenberg virus vectors, the *Culicoides* species (Diptera: Ceratopogonidae), in northern Europe

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Summary. Several species of *Culicoides* (Diptera: Ceratopogonidae) biting midges are biological vectors of bluetongue virus (BTV) and, as recently discovered, Schmallenberg virus (SBV) in northern Europe. Since their recent emergence in this part of the continent, these diseases that affect domestic and wild ruminants have caused considerable economic losses to the sheep and cattle industries. The substrates that are suitable for larval development of the main vector species are still relatively unknown. This study assessed all the substrates present in the immediate surroundings of a Belgian cattle farm and aimed to highlight the main breeding sites of these midge species. A total of 1639 immature *Culicoides* and 1320 adult specimens belonging to 13 species were found in 15 out of the 43 substrates studied: maize silage residues for *C. obsoletus/ C. scoticus*, old overwintered cattle dung in the meadow for *C. chiopterus* and *C. dewulfi*, ground of a flooded meadow, green filamentous algae and underlying substrate, silt from a pond, and ground of hollows caused by the crossing of machines on a dirt track for *C. festivipennis*, silt from a pond for *C. nubeculosus*, and ground of a flooded meadow for *C. lupicaris*. Identification of these micro-habitats and the associations among the species they contain could allow their localization and the development of new strategies of vector control, while preventing the creation of new *Culicoides* larval micro-habitats. Finally, measures designed to reduce larval populations could improve efficacy of vaccination campaigns against BTV in Europe.

Résumé. Sites de reproduction et association d'espèces de *Culicoides* (Diptera : Ceratopogonidae), principaux vecteurs en Europe du Nord des virus de la maladie de la langue bleue et de Schmallenberg. Plusieurs espèces de moucherons piqueurs du genre *Culicoides* (Diptera: Ceratopogonidae) jouent le rôle de vecteurs biologiques du virus de la Fièvre Catarrhale Ovine (FCO) et, comme découvert récemment, du virus de Schmallenberg (SB) en Europe du Nord. Depuis leur récente émergence dans cette partie du continent, ces maladies qui affectent les ruminants domestiques et sauvages ont causé des pertes économiques considérables au sein des cheptels ovins et bovins. Les substrats propices au développement larvaire des principales espèces impliquées dans la transmission de ces virus sont encore assez méconnus. Cette étude considère l'ensemble des substrats présents aux environs immédiats d'une exploitation agricole bovine belge et vise à mettre en évidence les gîtes larvaires des principales espèces de ces moucherons piqueurs. Un total de 1.639 *Culicoides* immatures et 1.320 spécimens adultes appartenant à 13 espèces ont été trouvés dans 15 des 43 substrats étudiés: résidus d'ensilage de maïs pour *C. obsoletus/C. scoticus*, vieilles bouses bovines ayant passé l'hiver en prairie pour *C. chiopterus* et *C. dewulfi*, terre d'une prairie inondée, algues vertes filamenteuses et terre sous-jacente, vase en bord de mare et terre du chemin de passage des machines pour *C. festivipennis*, vase en bord de mare pour *C. nubeculosus* et terre d'une prairie inondée pour *C. lupicaris*. L'identification de ces micro-habitats et des associations d'espèces qu'ils renferment permettra de les localiser et de développer de nouvelles stratégies de contrôle, tout en prévenant la création de nouveaux gîtes à risque. Des mesures visant à réduire les populations de larves pourraient finalement améliorer l'efficacité des campagnes de vaccination menées en Europe contre le virus de la FCO.

Bluetongue (BT) is a viral disease first described in South Africa (Hutcheon 1902) that affects domestic and wild ruminants. Serotype 8 recently emerged in northern Europe (World Organization for Animal Health 2006; Losson et al. 2007; Saegerman, Berkvens & Mellor 2008), including Belgium, in August 2006. During that year, approximately 2000 cases of BT were recorded in Europe, including 695 in Belgium (399 in sheep and 296 in bovines). For the year 2007 – and according to the information given by Federal Agency for the Safety of the Food Chain – Belgium had 6870 farms affected by this disease (4457 in cattle, 2400 in sheep and 13 in goat) out

of a total of 41000 outbreaks reported in Europe by the Notification System Animal disease of the European Commission. Increased rates of mortality and morbidity were recorded in 2007 (Szmaragd et al. 2007; Saegerman, Berkvens, Mellor, Dal Pozzo, et al. 2008). During 2008 and subsequent years, Bluetongue continued to spread throughout Europe. Economic losses related to serotype 8 of the BT virus (BTV) were considerable, although difficult to assess (Saegerman, Berkvens, Mellor, Dal Pozzo, et al. 2008). These losses were indeed both direct (e.g. death, infertility, abortions, stillbirths and congenital anomalies, weight loss and reduction in milk yield) and indirect (e.g. restrictions for live animal movement, their semen and some other products). The costs of preventive and control measures should also be taken into account (Sperlova & Zendulkova 2011). In 2011, a new virus that also affects ruminants was identified in northern Europe: Schmallenberg virus (SBV) (Hoffmann et al. 2012). SBV causes decreased milk production, diarrhoea and fever in adult cattle (Hoffmann et al. 2012), severe congenital malformations (mainly of the limbs, neck and brain) in lambs, calves and goat kids (Herder et al. 2012; Van den Brom et al. 2012), as well as abortions and stillbirths.

The biological vectors of BTV and SBV are biting midges belonging to the genus *Culicoides* Latreille 1809 (Du Toit 1944; De Regge et al. 2012; Veronesi et al. 2013). These dipterans, of a size ranging between 1 and 4 mm, can be found from the tropics to the tundra, and at altitudes of up to nearly 4000 m asl. For most species, the females are haematophagous, but only a few species may act in the propagation of these diseases (Delécolle & de La Rocque 2002; Mehlhorn et al. 2007; Carpenter et al. 2008; Meiswinkel et al. 2008; Saegerman, Berkvens & Mellor 2008; Hoffmann et al. 2009). Biting midges are also a source of nuisance through the bites of females. Their presence can therefore hinder the economic development of some regions, hampering agricultural and forestry activities as well as tourism development (Hendry & Godwin 1988). The egg laying and larval development happens preferentially in the uppermost layer (Uslu & Dik 2006) of semi-aquatic or wet substrates, which are rich in organic debris. The larvae eat organic matter or organisms such as nematodes, bacteria or protozoa (Chaker 1983). Although some major European *Culicoides* species breeding sites have been identified (Kettle & Lawson 1952; Murray 1957; Buxton 1960; Kremer et al. 1978; Uslu & Dik 2006, 2007; Glushchenko & Mirzaeva 2008; Zimmer et al. 2008, 2010; Kirkeby et al. 2009; Foxi & Delrio 2010; Harrup et al. 2013; Zimmer, Saegerman, et al. 2013), many unknown factors still remain about the ecology of the immature stages. In fact, the breeding sites of numerous *Culicoides* species – which include the main suspected vector species – are still poorly known in northern Europe. Thus, this justifies the need to conduct a general study on a cattle farm and all of its substrates.

This work – part of the results of which has already been briefly published as a letter communication (Zimmer et al. 2008) – assessed 43 substrates located within a Belgian cattle farm and a nearby meadow to identify larval micro-habitats of the main (potential) vector species of the BTV and the SBV in northern Europe.

Material and methods

Study sites

This study was conducted between March and July 2007 in Grand-Manil on a cattle farm named "Bedauwe farm," characterized by an open cowshed (50°33′19″ N, 4°41′06″ E) and in a neighbouring meadow (50°33′08″ N, 4°40′50″ E) located about 400 m from the farm. This village, with an essentially rural landscape, which spreads across 609 ha, is situated near Gembloux, in the province of Namur (Belgium). With a general uneven relief, the altitude varies between 130 and 165 m. The study sites selected (Figure 1) are situated on the course of the Orneau, the main river of Grand-Manil, which provides ideal conditions for the larval development of *Culicoides*. With an oceanic and mild climate, the territory of Grand-Manil is covered with a thick muddy layer, except for the banks of the Orneau, which has a stony ground charged with schists.

Sample selection and techniques

This study assessed all the substrates observed in the immediate surroundings of the studied cattle farm. A total of 43 different substrates were sampled in the meadow (ground of hollows caused by the crossing of machines on a dirt track and water in these hollows; vegetated ground of a dirt track; cattle dung and underlying ground; ground of a flooded meadow; ground of a livestock trampling area; moss on trees and on the ground; ground of an area with nettles; water from a ditch; ground under the stones and under a shed; silt and water from a pond; silt from a river; grass clods and ground located between them; decaying wood and sawdust; ravine ground; green filamentous algae and underlying ground; liquid and superficial ground of a ferrous flow; molehill ground; and their variants) and near the farm (used litter inside cowshed; water in stored tires; maize silage residues; greenish plaque of algae; manure; leftover feed; water of cattle trough; trampled ground; and their variants). For each substrate observed at the cattle farm level and at the nearby meadow level, two independent 2-litre samples were collected from a maximum depth of 10 cm, using a small garden shovel. Each of these samples was composed of four 0.5-litre samples in order to ensure their homogeneity. The samples were not sealed or placed in direct sunlight. This sampling was conducted three times during the study period: mid-March, at the end of April and mid-June. Back at the laboratory, each 2-litre sample of substrate was divided into two equal parts: the first one was used to collect the immature stages by an extraction technique, whereas the second one was incubated to allow the development of larvae into adults.

The extraction method adopted in this study was the direct flotation method. This technique, developed and adapted from Linley & Kettle (1964), Glukhova (1967) and Linley & Adams (1972), consisted of adding directly to each 1-litre sample a saturated saline solution ($MgSO_4$ in this study) with a density ranging between 1.17 and 1.20 kg m^{-3}. It allowed the larvae and pupae moving toward the surface to be collected with a clamp,
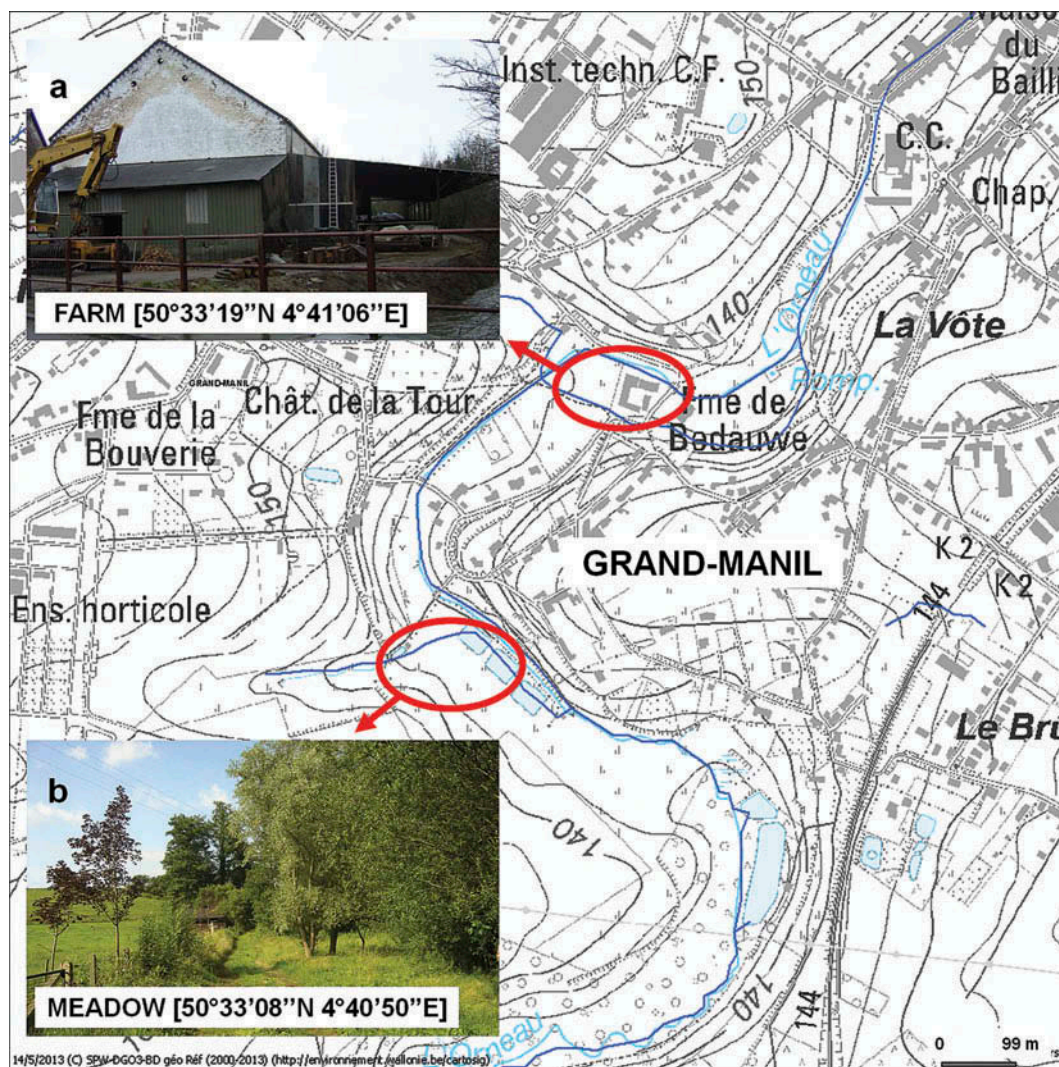
Figure 1. Localization of **a,** the 'Bedauwe farm' and **b,** its neighbouring meadow (Grand-Manil) (Map Source: SPW – DGARNE).

while the debris was sedimenting. The immature stages of *Culicoides* were then stored into a solution of 80% ethanol, sorted based on the general key of Glukhova (1977), counted under a binocular microscope (10–40× magnification). To facilitate the collection, this extraction was performed into wide-mouth containers.

The incubation method was adapted from Kremer et al. (1974). It consisted of placing the second 1-litre sample of each substrate – without any treatment – in a container with aeration and a central recovery system. These containers were then incubated at a temperature of 24°C (±2°C) using a thermostat during a four week period. The adults that hatched went up towards the light and could thus be harvested easily by means of a mouth-operated aspirator. The *Culicoides* adults that were daily captured were preserved in a solution of 80% ethanol, sorted using a stereomicroscope (10–40× magnification), sexed and then identified to species level using the morphological key of Delécolle (1985). However, *C. obsoletus* (Meigen 1818) and *C. scoticus* Downes & Kettle 1952 females could not be morphologically distinguished with certainty and were therefore classified as the Obsoletus complex.

Statistics

The results of the incubation technique were subjected to a multivariate statistical analysis based on principal components analysis (PCA) using Minitab® 15 software (Minitab Inc. 2006). This method transforms correlated variables (the *Culicoides* species here) into new ones that are independent of one another. These new variables are called "principal components" and constitute axes corresponding to the associations between the *Culicoides* species. This multivariate descriptive analysis placed the studied substrates in this system of axes in order to link the main *Culicoides* species (identified by the incubation method) to their respective breeding sites.

Results

The direct flotation technique

According to the direct flotation technique – only performed for the first two samplings – *Culicoides* larvae were found in seven out of the 43 substrates studied;

Table 1.   Number of *Culicoides* larvae and pupas collected by direct flotation during the first two samplings performed on the farm and meadow (Grand-Manil).

Substrates	Sample	Number of larvae	Number of pupae
Water of hollows caused by the crossing of machines, on a dirt track (meadow)	I	0	1
Old overwintered cattle dung in the meadow	I	66	2
	II	43	9
Ground of a flooded meadow	I	4	2
	II	1	1
Ground situated between two grass clods (meadow)	I	3	0
	II	8	0
Ground situated under green filamentous algae (meadow)	I	158	129
	II	239	51
Liquid (superficial ground) of a ferrous flow (meadow)	I	7	2
	II	1	0
Maize silage residues situated on the ground (farm)	I	336	91
	II	216	269

they were mainly wet edaphic and anthropogenic substrates (Table 1). Of these, three major breeding sites – with more than 100 *Culicoides* specimens for the first two samplings – emerged: old cattle dung and ground under algae from the meadow, and maize silage residues from the farm. In this study, the latter was the best breeding site for *Culicoides* (Figures 2–4). The direct flotation technique allowed the harvest of a total of 1639 *Culicoides* larvae and nymphs: 845 larvae and 531 pupas for the first sampling; 237 larvae and 26 pupas for the second. No *Culicoides* larvae were found in dry substrates.

The incubation technique

According to the incubation technique, 15 substrates were suitable for the development of *Culicoides*. Four of them contained a large number (>100 specimens) of *Culicoides*. These were, in order of importance: ground of a flooded meadow, maize silage residues, ground under algae and old cattle dung. No *Culicoides* specimens emerged from dry substrates. With the incubation method, a total of 1320 adult *Culicoides* specimens belonging to 13 species were identified for the different wet substrates. These *Culicoides* were, in order of importance: *Culicoides festivipennis* Kieffer 1914, *C. obsoletus/C. scoticus*, *Culicoides chiopterus* (Meigen 1830), *Culicoides dewulfi* Goetghebuer 1936, *Culicoides nubeculosus* (Meigen 1830), *Culicoides lupicaris* Downes & Kettle 1952, *Culicoides pulicaris* (L. 1758), *Culicoides stigma* (Meigen 1818), *Culicoides salinarius* Kieffer 1914, *Culicoides circumscriptus* Kieffer 1918, *Culicoides kibunensis* Tokunaga 1937 and *Culicoides puncticollis* (Becker 1903). It should be noted that only the first four species were abundant (> 100 specimens), whereas the others were present in relatively small quantities (Table 2).

Figure 2.   Larvae and nymphs floating toward the surface during the direct flotation assay in a sample (1 l) of maize silage residues (Grand-Manil).
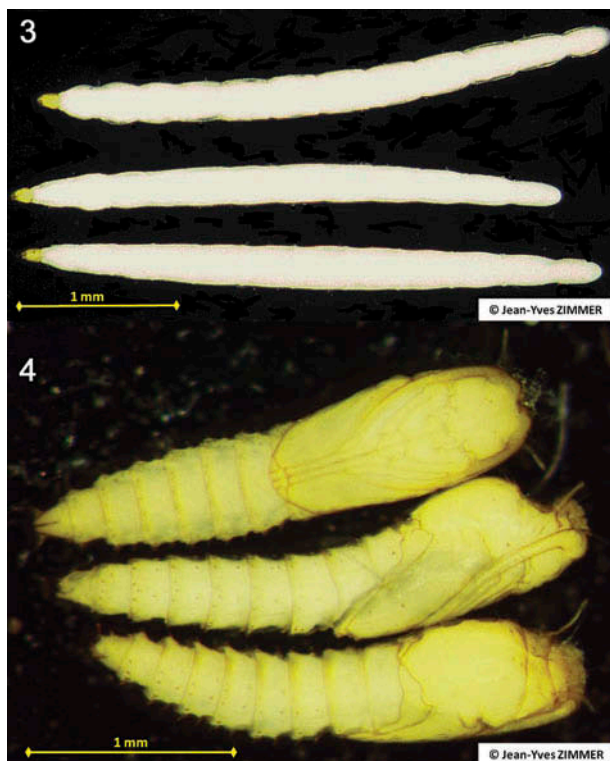
Figures 3–4. **3,** Larvae and **4,** pupae extracted by direct flotation from a sample (1 l) of maize silage residues (Grand-Manil).

The total number of *Culicoides* obtained during the various samplings decreased between March and June: 806 specimens (of 10 species) were procured by the first sampling, 469 (of seven species) by the second and 45 (of six species) by the last one. Moreover, we observed that all specimens of *C. chiopterus* and *C. dewulfi* came from the first sampling of the substrates (except one *C. chiopterus* that was obtained by the second sampling). The immature stages of the Obsoletus complex also appeared to be more abundant in mid-March (85%) than at the end of April (14%) or mid-June (1%). The proportion of males obtained by the two first samplings was identical (58%), but lower for the last sampling (31%).

Considering the presence of a significant variability of the incubation results and of a large quantity of individuals of certain species at some substrates, it was preferable not to standardize the variables using a matrix of covariance in the PCA. According to the PCA performed on the mid-March sampling, the first three components explain 99.7% of the total variation. The species that most contribute to an explanation for the variation is *C. obsoletus/C. scoticus* in PC1, *C. festivipennis* in PC2, and *C. chiopterus* and *C. dewulfi* in PC3. Charts of the studied substrates in the factorial plans PC1 and PC2, as well as in PC1 and PC3, are presented in Figure 5a, b. The chart in the plans PC2 and PC3 is similar.

According to the PCA performed on the end-of-April sampling, the first two components explain 100% of the total variation. The species that most contribute to an explanation for the variation is *C. festivipennis* in PC1, and *C. obsoletus/C. scoticus* in PC2. A chart of the studied substrates in the first two factorial plans is presented in Figure 5c.

According to the PCA performed on the mid-June sampling, the first two components explain 97.8% of the total variation. The species that most contribute to an explanation for the variation is *C. festivipennis* in PC1, and *C. nubeculosus* in PC2. A chart of the studied substrates in the first two factorial plans is presented in Figure 5d.

The general shape of these graphs revealed a relationship of mutual exclusion between the Obsoletus complex and *C. festivipennis*, as well as between *C. obsoletus/ C. scoticus* and the *C. chiopterus/C. dewulfi* species or between *C. festivipennis* and the *C. chiopterus/C. dewulfi* species. This analysis highlighted the larval microhabitats of some species and the following associations: *C. obsoletus/C. scoticus* were associated with maize silage residues, *C. festivipennis* with the ground of a flooded meadow, green filamentous algae, and the ground situated under these algae, *C. chiopterus* and *C. dewulfi* with old overwintered cattle dung, and *C. nubeculosus* with the silt from a pond.

Discussion

The direct flotation technique confirmed that *Culicoides* larvae avoid drought and colonize the superficial layer of some wet substrates that are not permanently immersed, as has been previously shown by Rieb (1982), Chaker (1983), and Uslu & Dik (2006). Nonetheless, it should be noted that this method is mainly suitable for samples with large larvae (stages III and IV), which are easier to locate, and pupae; the identification of the collected immature stages remains particularly complicated.

Incubation at 24°C provides more complete results than the direct flotation technique. In fact, not only does it indicate a greater number of larval micro-habitats, but it also allows an easier identification of the species present. In addition, the method is performed at a higher temperature than that found at a natural habitat, which accelerates larval development and thus allows foresight into the massive emergences occurring in nature. The number of *Culicoides* from the substrate samples decreased between the samplings made between March and June. This can be explained by the progressive development of larvae and their emergence as adults, thereby reducing the immature population. A higher proportion of pupae obtained by direct flotation from the first sampling (mid-March) confirms this. Overwintering and aestivalhibernation of *Culicoides* larvae (Rieb 1987) further justifies the large presence of the immature stages in their respective breeding sites early in the year. The species *C. chiopterus*

Table 2. Substrates suitable for the development of *Culicoides* and details of the respective species obtained by the three samplings (according to the incubation method) performed in Grand-Manil.

| Substrate	*Culicoides* species											
	chiopterus	*circumscriptus*	*dewulfi*	*festivipennis*	*kibunensis*	*lupicaris*	*nubeculosus*	*obsoletus/ scoticus*	*pulicaris*	*puncticollis*	*salinarius*	*stigma*
Ground of hollows caused by the crossing of machines, on a dirt track (with water) (no. 1)	0	0	0	7	0	0	0	0	0	1	2	0
Ground of hollows caused by the crossing of machines, on a dirt track (without water) (no. 2)	0	0	0	4	0	0	1	0	0	0	0	1
Old overwintered cattle dung in the meadow (no. 4)	133	0	82	0	0	0	0	0	0	0	0	0
Ground under the old cattle dung (no. 5)	23	0	19	0	0	0	0	0	0	0	0	0
Ground of a flooded meadow (no. 7)	1	1	0	355	0	11	0	1	1	0	1	1
Silt from a pond (in a light slope → out of water) (no. 16)	0	0	0	11	0	1	11	1	1	0	0	1
Silt from a pond (in water) (no. 17)	0	0	0	30	0	0	9	0	0	0	1	1
Grass clod in the meadow (no. 19)	0	0	0	0	0	0	0	0	4	0	0	0
Ground situated between two grass clods (no. 20)	0	0	0	1	0	0	0	0	0	0	0	4
Amount of green filamentous algae in the meadow (no. 27)	0	0	0	23	0	0	0	0	0	0	0	0
Ground situated under green filamentous algae (no. 28)	0	0	0	228	2	9	1	0	2	0	0	0
Liquid (superficial ground) of a ferrous flow (no. 29)	0	0	0	8	0	1	1	0	0	0	0	0
Maize silage residues situated on the ground near the farm (no. 37)	1	0	8	0	0	0	0	311	0	0	0	0
Greenish plaque (algae) situated on the ground near the farm (no. 39)	0	1	0	0	0	0	2	0	0	0	0	0
Ground of an area affected by cattle and machines (no. 43)	1	0	0	0	0	0	0	0	0	0	0	0
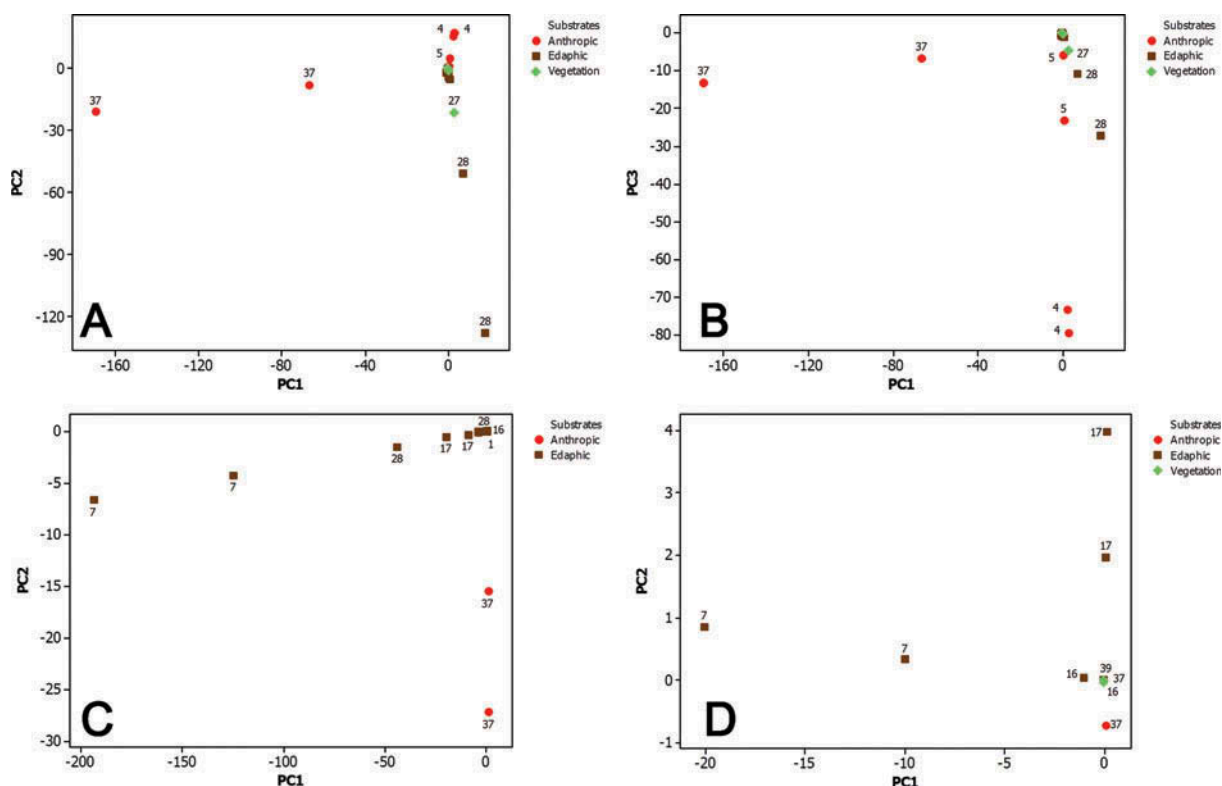
Figure 5.   Charts of the PCA of the **A, B,** mid-March, **C,** end of April and **D,** mid-June samplings giving the studied substrates in the considered factorial plans. (Substrate identification: no. 4, old cattle dung; no. 5, ground under old cattle dung; no. 7, ground of a wet meadow; no. 17, silt from a pond; no. 27, green algae; no. 28, ground under green algae; and no. 37, maize silage residues.)

and *C. dewulfi* were found almost exclusively during the first sampling in old overwintered cattle dung located in the meadow. This micro-habitat quickly degrades over time, and hence seems to be colonized less by *Culicoides* larvae. However, in another study conducted in parallel at the same site and involving the light trapping of adult *Culicoides*, almost 99% of the specimens of both species were captured from the farm (Zimmer et al. 2009). These two species can migrate from their breeding sites (old cattle dung located in the meadow) to livestock (opened cowshed). The ability to follow livestock inside the cowshed has already been suggested for *C. obsoletus* (Meiswinkel et al. 2008).

Examination of the charts generated by PCA allows the description of micro-habitats and species association of the main (potential) vectors of BTV and SBV, namely *C. dewulfi, C. chiopterus, C. obsoletus/C. scoticus* and *C. nubeculosus*. The latter especially looked for a semi-aquatic edaphic substrate; indeed, it prefers silt from a pond. The first four species however prefer substrates associated with human and livestock: *C. dewulfi* and *C. chiopterus* were found, for example, in overwintered cattle dung on the meadow, as previously observed (Kettle & Lawson 1952; Kettle 1962). The two species of the Obsoletus complex – which are involved in the transmission of BTV serotype 8 and SBV (Carpenter et al. 2008;

Hoffmann et al. 2009; De Regge et al. 2012) and constitute the primary complex in northern Europe near to farms (Zimmer et al. 2009; Zimmer, Smeets, et al. 2013) – were found in the maize silage residues situated on the ground near the farm (Figure 6). The major larval habitats of this complex were unknown before this study, which highlighted for the first time the importance of silage residues as *C. obsoletus/C. scoticus* breeding sites (Zimmer et al. 2008); compost heaps of leaves and tree holes have been previously reported as secondary breeding sites for *C. obsoletus* (Murray 1957; Chaker 1983), as well as straw contaminated with faeces and manure piles (Weinburgh & Pratt 1962). Wet forest litter appeared also suitable for the larval development of the *C. obsoletus* group members (Glushchenko & Mirzaeva 2008), as well as stagnant water reservoirs and marshes with dense vegetation (Dzhafarov 1976). Moreover, Buxton (1960) reared *C. scoticus* from large rotting fungi. A recent study showed that the larvae of this complex were also found directly inside the stables, and in dried cattle dung stuck to the walls resulting from the partial removal of surface litter (Zimmer et al. 2010). The immature stages of *C. festivipennis* proliferated in most of the semi-watery substrates such as ground of a flooded meadow, ground under green algae, silt from a pond, and ground of hollows

Figure 6.   Maize silage residues revealing the presence of the two species of the Obsoletus complex in Grand-Manil (Belgium).

caused by the crossing of machines on a dirt track. Therefore, this species grows in different breeding sites and presents a strong ability to adapt, as *C. circumscriptus* and *C. puncticollis* (Uslu & Dik 2007). The species *C. festivipennis* was so previously observed in mud samples along a pond shoreline (Foxi & Delrio 2010), in bare mud without vegetation associated with *C. stigma*, *C. nubeculosus* and *C. circumscriptus* (Kettle & Lawson 1952) and in swamps with the water level above the soil surface associated with *C. pulicaris* and *C. punctatus* (Kettle & Lawson 1952). These two last species were also collected in samples of a wet grazed field with manure (Kirkeby et al. 2009) and *C. kibunensis* in eutrophic fresh water marshes (Kettle & Lawson 1952). The species *C. lupicaris* was observed in ground of a flooded meadow. Some micro-habitats appear to house a variety of different *Culicoides* species: the ground of a flooded meadow (eight species), silt from a pond (six species) and the ground under green filamentous algae (five species). Nevertheless, *C. festivipennis* was found to be the major species occupying each of them. The absence of *Culicoides impunctatus* Goetghebuer 1920 during this study could be explained by its preference for bogs (Zimmer, Smeets, et al. 2013), characterized by oligotrophic soils.

The abundance of males obtained by the incubation method (58% for the two first samplings) compared to females can be explained in different ways: a naturally higher proportion of males, a very early emergence of females, a greater sensitivity of female larvae to manipulations suffered by the substrate or an influence of rearing temperature on the sex ratio. This latter factor has been demonstrated by Bishop et al. (1996) for *Culicoides brevitarsis* Kieffer 1917; the males of this species were dominant at low temperatures and the females at high temperatures. In Turkey, Uslu & Dik (2010) observed almost 45% of males during a study using a quite similar technique of Culicoides emergence in the laboratory.

The massive outbreaks observed during the same week for many of the substrates indicate that the larvae are distributed in aggregates of developmental stages. In addition, the time of emergence differed among the species, further suggesting that the larvae are distributed in aggregates of species, as suggested by Rieb (1982). The cohabitation of both aggregates constitutes the observed species association. The level of ecological requirement for each species could explain how larvae of some species cohabit in the same substrate. Physicochemical characteristics of micro-habitats might further demonstrate why certain substrates are preferred by adult females or larvae over others.

Conclusion

This study highlights that the breeding sites of some of the major biting midge species involved in the transmission of BTV and SBV are found in large numbers in some anthropogenic substrates, close to farms and nearby meadows: Obsoletus complex in maize silage residues (first major breeding site for these important species), *C. chiopterus* and *C. dewulfi* in old overwintered cattle dung and *C. nubeculosus* in the silt from a pond. A non-vector species of BTV (*C. festivipennis*) occurs in a wide range

of wetlands (ground of a flooded meadow, ground under green algae, silt from a pond, and ground of hollows caused by the crossing of machines on a dirt track). Some species have a strong ability to adapt and so present a plasticity in habitat utilization (*C. festivipennis*), but some others seem environmentally more demanding (*C. chiopterus* and *C. dewulfi*). Moreover, some species seem to have similar ecological requirements and, therefore, occur at similar breeding sites (*C. chiopterus* and *C. dewulfi*), while others are generally not observed together in the same anthropogenic substrate (*C. obsoletus/C. scoticus* and *C. festivipennis*; *C. obsoletus/C. scoticus* and *C. chiopterus/C. dewulfi*; *C. festivipennis* and *C. chiopterus/C. dewulfi*).

The incubation technique accelerates larval development, and therefore enables foresight into the emergence of *Culicoides* in the field. This method could be useful in developing preventive measures against the vectors of BTV and SBV.

The identification and characterization of *Culicoides* breeding sites will assist with the location and the development of new strategies of vector control, while preventing the creation of new suitable larval micro-habitats. Hygienic measures on farms, such as the reduction of silage residues and treatment with methods such as composting and acidification, could reduce biting midge populations and assist with control of these emerging diseases in Europe.

References

Bishop AL, McKenzie HJ, Barchia IM, Harris AM. 1996. Effect of temperature regimes on the development, survival and emergence of *Culicoides brevitarsis* Kieffer (Diptera: Ceratopogonidae) in bovine dung. Australian Journal of Entomology. 35:361–368.

Buxton PA. 1960. British Diptera associated with fungi. III. Flies of all families reared from about 150 species of fungi. Entomologists' Monthly Magazine. 96:61–94.

Carpenter S, McArthur C, Selby R, Ward R, Nolan DV, Mordue Luntz AJ, Dallas JF, Tripet F, Mellor PS. 2008. Experimental

infection studies of UK *Culicoides* species midges with bluetongue virus serotypes 8 and 9. Veterinary Record. 163:589–592.

Chaker E. 1983. Contribution à l'étude de la morphologie et de la diagnose des larves de Culicoides (Diptera, Ceratopogonidae) [thèse de Doctorat es Sciences Pharmaceutiques (Diplôme d'Etat)]. Université Louis Pasteur de Strasbourg, no. 56, 229 p.

Delécolle JC. 1985. Nouvelle contribution à l'étude systématique et iconographique des espèces du genre Culicoides (Diptera, Ceratopogonidae) du Nord-Est de la France [thèse de Doctorat d'Université]. Université Louis Pasteur de Strasbourg, no. 293, 238 p.

Delécolle JC, de La Rocque S. 2002. Contribution à l'étude des *Culicoides* de Corse. Liste des espèces recensées en 2000/2001 et redescription du principal vecteur de la fièvre catarrhale ovine : *C. imicola* Kieffer, 1913 (Diptera, Ceratopogonidae). Bulletin de la Société entomologique de France. 107:371–379.

De Regge N, Deblauwe I, De Deken R, Vantieghem P, Madder M, Geysen D, Smeets F, Losson B, van den Berg T, Cay AB. 2012. Detection of Schmallenberg virus in different *Culicoides* spp. by real-time RT-PCR. Transboundary and Emerging Diseases. 59:471-475.

Du Toit RM. 1944. The transmission of blue-tongue and horse sickness by *Culicoides*. Onderstepoort Journal of Veterinary Science and Animal Industry. 19:7–16.

Dzhafarov SM. 1976. Biting midges (Diptera: Heleidae) of Transcaucasus (morphology, biology, ecology, geographical distribution and harmfulness, control, fauna of the genera *Culicoides, Leptoconops* and *Lasiohelea*). Cairo: Franklin Book Programs. 525 p.

Foxi C, Delrio G. 2010. Larval habitats and seasonal abundance of *Culicoides* biting midges found in association with sheep in northern Sardinia, Italy. Medical and Veterinary Entomology. 24:199–209.

Glukhova VM. 1967. On the technique of collecting and cultivation of larvae of bloodsucking midges (Diptera, Ceratopogonidae). Parazitologiya. 1:171–175.

Glukhova VM. 1977. On the subgenera classification of the genus *Culicoides* Latreille 1809 (Diptera, Ceratopogonidae) considerating morphological structures of larvae. Parazitologiya. 27:112–118.

Glushchenko NP, Mirzaeva AG. 2008. Larval habitats and species composition of bloodsucking midges (Diptera: Ceratopogonidae) in mountain landscapes of South Siberia and the Russian Far East. Russian Entomological Journal. 17:63–70.

Harrup LE, Purse BV, Golding N, Mellor PS, Carpenter S. 2013. Larval development and emergence sites of farm-associated *Culicoides* in the United Kingdom. Medical and Veterinary Entomology. doi:10.1111/mve.12006.

Hendry G, Godwin G. 1988. Biting midges in Scottish forestry: a costly irritant or a trivial nuisance? Scottish Forestry. 42: 113–119.

Herder V, Wohlsein P, Peters M, Hansmann F, Baumgärtner W. 2012. Salient lesions in domestic ruminants infected with the emerging so-called Schmallenberg virus in Germany. Veterinary Pathology. 49:588–591.

Hoffmann B, Bauer B, Bauer C, Bätza H-J, Beer M, Clausen P-H, Geier M, Gethmann JM, Kiel E, Liebisch G, Liebisch A, Mehlhorn H, Schaub GA, Werner D, Conraths FJ. 2009. Monitoring of putative vectors of bluetongue virus serotype 8, Germany. Emerging Infectious Diseases. 15:1481–1484.

Hoffmann B, Scheuch M, Höper D, Jungblut R, Holsteg M, Schirrmeier H, Eschbaumer M, Goller KV, Wernike K, Fischer M, Breithaupt A, Mettenleiter TC, Beer M. 2012. Novel Orthobunyavirus in cattle, Europe, 2011. Emerging Infectious Diseases. 18:469–472.

Hutcheon D. 1902. Malarial catarrhal fever of sheep. Veterinary Record. 14:629–633.

Kettle DS. 1962. The bionomics and control of *Culicoides* and *Leptoconops* (Diptera, Ceratopogonidae = Heleidae). Annual Review of Entomology. 7:401–418.

Kettle DS, Lawson JWH. 1952. The early stages of British biting midges *Culicoides* Latreille (Diptera: Ceratopogonidae) and allied genera. Bulletin of Entomological Research. 43:421–467.

Kirkeby C, Bodker R, Stockmarr A, Enoe C. 2009. Association between land cover and *Culicoides* (Diptera: Ceratopogonidae) breeding sites on four Danish cattle farms. Entomologica Fennica. 20:228–232.

Kremer M, Hirtzel C, Molet B. 1974. Résultats préliminaires d'une étude écologique des *Culicoides* de Sansouires de Camargue. Annales de Parisitologie Humaine et Comparée. 49:653–656.

Kremer M, Rieb J-P, Rebholtz C. 1978. Ecology of the cerato-pogonids of the Alsace plain. I. The genus *Culicoides* in the humid soils of the Ried. Annales de Parasitologie Humaine et Comparée. 53:101–115.

Linley JR, Adams GM. 1972. Ecology and behaviour of imma-ture *Culicoides melleus*. Bulletin of Entomological Research. 62:113–127.

Linley JR, Kettle DS. 1964. A description of the larvae and pupae of *Culicoides furens* Poey, and *Culicoides hoffmani* Fox (Diptera, Ceratopogonidae). Annals and Magazine of Natural History. 7:129–149.

Losson B, Mignon B, Paternostre J, Madder M, De Deken R, De Deken G, Deblauwe I, Fassotte C, Cors R, Defrance T, Delécolle J-C, Baldet T, Haubruge E, Frédéric F, Bortels J, Simonon G. 2007. Biting midges overwintering in Belgium. Veterinary Record. 160:451–452.

Mehlhorn H, Walldorf V, Klimpel S, Jahn B, Jaeger F, Eschweiler J, Hoffmann B, Beer M. 2007. First occurrence of *Culicoides obsoletus*-transmitted Bluetongue virus epidemic in Central Europe. Parasitology Research. 101:219–228.

Meiswinkel R, Baldet T, De Deken R, Takken W, Delécolle JC, Mellor PS. 2008. The 2006 outbreak of bluetongue in north-ern Europe – The entomological perspective. Preventive Veterinary Medicine. 87:55–63.

Minitab Inc. 2006. Minitab® Statistical Software, Release 15 for Windows. State College, PA, USA.

Murray WS. 1957. Investigations on the bionomics of *Culicoides obsoletus* (Meigen) and other biting midges at Mount Solon, Virginia. Mosquito News. 17:77–82.

Rieb J-P. 1982. Contribution à la connaissance de l'écologie et de la biologie des Cératopogonidés (Diptera, Nematocera) [thèse d'Université]. Strasbourg (France): Université Louis Pasteur de Strasbourg, 395 p.

Rieb J-P. 1987. L'estivo-hibernation et le contrôle de la dynami-que du cycle évolutif dans le genre *Culicoides* (Diptères, Cératopogonidés). Vie Milieu. 37:23–37.

Saegerman C, Berkvens D, Mellor PS. 2008. Bluetongue epide-miology in the European Union. Emerging Infectious Diseases. 14:539–544.

Saegerman C, Berkvens D, Mellor PS, Dal Pozzo F, Porter S, Zientara S. 2008. Fièvre catarrhale ovine: l'Europe au carre-four de l'enzootie. Point Vétérinaire. 290:41–47.

Sperlova A, Zendulkova D. 2011. Bluetongue: a review. Veterinarni Medicina. 56:430–452.

Szmaragd C, Wilson A, Carpenter S, Mertens PPC, Mellor PS, Gubbins S. 2007. Mortality and case fatality during the recurrence of BTV-8 in northern Europe in 2007. Veterinary Record 161:571–572.

Uslu U, Dik B. 2006. Vertical distribution of *Culicoides* larvae and pupae. Medical and Veterinary Entomology. 20:350–352.

Uslu U, Dik B. 2007. Description of breeding sites of *Culicoides* species (Diptera: Ceratopogonidae) in Turkey. Parasite. 14: 173–177.

Uslu U, Dik B. 2010. Chemical characteristics of breeding sites of *Culicoides* species (Diptera: Ceratopogonidae). Veterinary Parasitology. 169:178–184.

Van den Brom R, Luttikholt SJ, Lievaart-Peterson K, Peperkamp NH, Mars MH, van der Poel WH, Vellema P. 2012. Epizootic of ovine congenital malformations associated with Schmallenberg virus infection. Tijdschrift voor Diergeneeskunde. 137:106–111.

Veronesi E, Henstock M, Gubbins S, Batten C, Manley R, Barber J, Hoffmann B, Beer M, Attoui H, Mertens PPC, Carpenter S. 2013. Implicating *Culicoides* biting midges as vectors of Schmallenberg virus using semi-quantitative RT-PCR. PLoS ONE. 8: e57747. doi:10.1371/journal.pone.0057747.

Weinburgh HB, Pratt HD. 1962. *Culicoides*, public health impor-tance, biology, survey, and control. US Department of Health, Education and Welfare, Public Health Service, Communicable Disease Center, Atlanta, Georgia.

World Organization for Animal Health. 2006. Bluetongue detected for the first time in Northern Europe. Press release. Aug 23. Available from http://www.oie.int/eng/press/en_060823.htm

Zimmer J-Y, Haubruge E, Francis F, Bortels J, Simonon G, Losson B, Mignon B, Paternostre J, De Deken R, De Deken G, Deblauwe I, Fassotte C, Cors R, Defrance T. 2008. Breeding sites of bluetongue vectors in northern Europe. Veterinary Record. 162:131.

Zimmer J-Y, Losson B, Saegerman C, Haubruge E. 2009. Ecologie et distribution des espèces de *Culicoides* Latreille 1809 (Diptera : Ceratopogonidae) à proximité d'une exploi-tation bovine en Belgique. Annales de la Société entomolo-gique de France. 45:393–400.

Zimmer J-Y, Saegerman C, Losson B, Beckers Y, Haubruge E, Francis F. 2013. Chemical composition of silage residues sustain-ing the larval development of the *Culicoides obsoletus/Culicoides scoticus* species (Diptera: Ceratopogonidae). Veterinary Parasitology. 191:197–201.

Zimmer J-Y, Saegerman C, Losson B, Haubruge E. 2010. Breeding sites of bluetongue virus vectors, Belgium. Emerging Infectious Diseases. 16:575–576.

Zimmer J-Y, Smeets F, Simonon G, Fagot J, Haubruge E, Francis F, Losson B, 2013. Are bogs reservoirs for emerging disease vectors? Evaluation of *Culicoides* populations in the Hautes Fagnes Nature Reserve (Belgium). PLoS ONE. 8: e66893. doi:10.1371/journal.pone.0066893.