One Health 16 (2023) 100572

Contents lists available at ScienceDirect

One Health

journal homepage: www.elsevier.com/locate/onehlt

Overwintering *Culex torrentium* in abandoned animal burrows as a reservoir for arboviruses in Central Europe

Felix G. Sauer [a,*], Unchana Lange [a], Jonas Schmidt-Chanasit [a,b], Ellen Kiel [c], Blanka Wiatrowska [d], Łukasz Myczko [e,1], Renke Lühken [a,1]

[a] *Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany*
[b] *Faculty of Mathematics, Informatics and Natural Sciences, University of Hamburg, Hamburg, Germany*
[c] *Aquatic Ecology and Nature Conservation, Carl von Ossietzky University, Oldenburg, Germany*
[d] *Department of Botany and Forest Habitats, Poznań University of Life Sciences, Poznań, Poland*
[e] *Department of Zoology, Poznań University of Life Sciences, Poznań, Poland*

ARTICLE INFO

Keywords:
Animal burrow
Culex torrentium
Overwintering
Poland
Usutu virus

ABSTRACT

Culex pipiens s.s./*Culex torrentium* belong to the most widespread mosquito taxa in Europe and are the main vectors of Sindbis, West Nile and Usutu virus. The adult overwintering females can act as reservoir for these arthropod-borne viruses (arboviruses), thus contributing to their local persistence when transmission cycles are interrupted during the winter. However, the main overwintering sites of *Cx. torrentium* are unknown.

In a study from 2017, 3455 *Cx. pipiens* s.s./*Cx. torrentium* specimens were collected from abandoned animal burrows in Poznan, Poland. These specimens were retrospectively identified to species-level with a PCR assay, which revealed *Cx. torrentium* as dominant species (> 60%). Motivated by these results, we conducted a field study from February to July 2022 to systematically analyse the overwintering site patterns of *Cx. pipiens* s.s./*Cx. torrentium*. Mosquitoes were sampled using pipe traps in abandoned animal burrows ($n = 20$) and with aspirators in nearby anthropogenic overwintering sites ($n = 23$). All *Cx. pipiens* s.s./*Cx. torrentium* were screened for *Flaviviridae* RNA.

In total, 4710 mosquitoes of five different taxa were collected from anthropogenic sites. 3977 of them were identified as *Cx. p. pipiens*/*Cx. torrentium* (*Cx. p. pipiens*: 85%, *Cx torrentium*: 1%, pools with both species: 14%). In contrast, only *Cx. p. pipiens*/*Cx. torrentium* (1688 specimens) were collected from animal burrows dominated by *Cx. torrentium* (52%), followed by pools with both species (40%) and *Cx. p pipiens* (8%). A single pool of 10 *Cx. torrentium* specimens collected from an animal burrow was positive for Usutu virus.

The detection of Usutu virus demonstrates that *Cx. torrentium* can act as winter reservoir for arboviruses. Abandoned animal burrows may by the primary overwintering site for the species and should be considered in future surveillance programmes, when sampling overwintering mosquitoes.

1. Introduction

Three *Culex* taxa are considered the most widespread mosquito species in Central Europe: *Culex. torrentium* Martini, 1925 and the two ecologically distinct forms of *Culex pipiens* s.s, *Culex pipiens* form *pipiens* (*Cx. p. pipiens*) Linnaeus, 1758 and *Cx. pipiens* form *molestus* (*Cx. p. molestus*) Forskal, 1775 [1]. The females of the three mosquito taxa cannot be differentiated with classical morphology [1]. However, the differentiation is important since each taxon has a different vector capacity for viruses. The hybrid with an intermediate host-feeding preference between *Cx. p. pipiens* (ornithophilic) and *Cx. p. molestus* (anthropophilic) is considered to play an important role as bridge vector for West Nile virus (WNV) in the United States [2]. While *Cx. pipiens* s.s. is widely distributed in the America, Europe, Asia, Africa and Australia [3], *Cx. torrentium* is predominantly restricted to Central and Northern Europe. This species has a much higher vector competence for WNV and Sindbis virus (SINV) compared to *Cx. p. pipiens* [4–6] and is also known as competent vector for Usutu virus (USUV) [7].

* Corresponding author.
E-mail address: felix.sauer@bnitm.de (F.G. Sauer).
[1] Shared last authorship.

https://doi.org/10.1016/j.onehlt.2023.100572
Received 23 February 2023; Received in revised form 22 May 2023; Accepted 25 May 2023
Available online 29 May 2023

The overwintering strategy of adult mosquitoes is an important facet of the overwintering mechanisms for arthropod-borne viruses (arboviruses) in temperate regions [8]. Overwintering mosquitoes can serve as virus reservoir when transmission cycles are interrupted by the low winter temperatures, resulting in reduced vector activity and virus amplification. Vertical transmission is considered to be an important mechanism for the infection of overwintering females [9], which was proven possible for different *Culex* species [10,11]. This was also confirmed by the detections of WNV in overwintering *Culex* specimens in the United States [12–14], the Czech Republic [15], Russia [16] or Germany [17].

While most native mosquito species, such as *Aedes* species, undergo overwintering the egg or larvae stage, *Cx. p. pipiens* and *Cx. torrentium* obligatory overwinter as adult females [18]. Different studies indicate distinct differences in the overwintering sites of *Cx. p. pipiens* and *Cx. torrentium*. Several studies found *Cx. p. pipiens* in man-made shelters such as cellars, mines and bunkers [19–21]. In contrast, *Cx. torrentium* is rarely detected in anthropogenic overwintering habitats [22], but studies before the 21th century must be interpreted with caution as a reliable differentiation of *Culex p. pipiens/Cx. torrentium* females is only possible with molecular techniques. Nevertheless, the underrepresentation of *Cx. torrentium* in cellars and aboveground buildings was also confirmed in recent studies [23–25]. As shown in vector competence studies, *Cx. torrentium* is probably one of the major vectors of WNV, SINV and USUV in central and northern Europe [5–7,26]. Knowledge on their primary overwintering sites will allow a more detailed understanding of the role of *Cx. torrentium* for the persistence of arboviruses in temperate regions and would facilitate an effective surveillance of this important vector species in winter.

In 2021, Myczko et al. [27] published a study on overwintering arthropods in the burrows of red foxes (*Vulpes vulpes*) and badgers (*Meles meles*). Thereby, by far the highest proportion of the arthropods were classified as *Cx. p. pipiens/Cx. torrentium* not further differentiated to the species level. In our study, retrospective molecular analysis of these samples confirmed a distinctly higher proportion of *Cx. torrentium* (> 60%). To further evaluate if there is a species-specific difference between the overwintering sites of *Cx. p. pipiens* and *Cx. torrentium*, we conducted a systematic study with paired-sample design, sampling overwintering mosquitoes from burrows and nearby anthropogenic overwintering sites.

2. Material and methods

The study was conducted in the region around the city of Poznan (52°24′29"N 16°56′01″E), western Poland. Burrows of foxes and badgers were located in forest patches predominantly dominated by Scots pine (*Pinus sylvestris*), while the surrounding area was characterised by a high proportion of arable land. For 2017, the selection process of 10 burrows is described in detail by Myczko et al. [27]. In 2022, five study areas with animal burrows of foxes and badgers were selected. Anthropogenic overwintering sites (cellars and abandoned aboveground buildings) in a minimum distance of 0.1 km and a maximum distance of 1 km from the burrows were sampled to directly compare the overwintering site patterns of *Cx. p. pipiens* and *Cx. torrentium*. At each study area, three to five animal burrows and three to five anthropogenic overwintering sites were investigated for overwintering mosquitoes. In total, 20 animal burrows and 23 anthropogenic overwintering sites were included in the study 2022.

Only abandoned animal burrows were sampled. If the absence of badgers and foxes was unclear, the burrow entrances were covered with wooden sticks for several days to check if mammals tried to enter or leave the burrows in the meantime. Photos of the entrance were taken for comparison between the controls. In addition, we also looked for paw prints in the fresh soil or snow in front of the burrow entrance. It is not always possible to clearly determine whether the abandoned burrow was dug by foxes or badgers. Sometimes both species even use the same [28,29]. Therefore, burrows of badgers and foxes were not further differentiated in this study. Mosquito sampling from the abandoned burrows was conducted with pipe traps [27]. Briefly, the traps consist of a PVC pipe with a diameter of 200 mm with one side closed by a mosquito net. The open side was pushed into the burrow entrance and the open edges on the side of the pipe were covered with soil. The arthropods flying out of the animal burrows were captured in a funnel inside the pipe.

In the study of 2017 [27], weekly samplings were conducted from the mid of March to the beginning of July, except for the last two controls carried out at a biweekly interval. Samples were stored in 75% alcohol. In 2022, the pipe traps were controlled weekly between 01.03.2022 and 05.07.2022, except for the last two controls carried out at a biweekly interval. In addition, nearby cellars and abandoned aboveground buildings were sampled between 14.02.2022 and 17.02.2022 with handmade aspirators similar to the model described by Vazquez-Prokopec et al. [30]. Strong flashlights were used to help locate the resting mosquitoes at the anthropogenic overwintering sites. Mosquitoes sampled from 2022 were killed by freezing and were stored at −24 °C until further analysis.

All specimens were identified by morphological characters to the lowest level of taxonomic certainty [1]. Additionally, *Cx. pipiens* s.s./*Cx. torrentium* specimens were typed to species level using the real-time PCR assay described by Rudolf et al. [31]. Thereby, a random subsample of 10% of the individuals was analysed individually. The remaining specimens were pooled by sampling site and sampling date with up to 10 specimens. All *Cx. pipiens* s.s./*Cx. torrentium* samples were screened for flaviviruses RNA with the pan-flavivirus RT-PCR modified from Chao et al. [32]. The amplicons were sequenced with LGC Genomics (Berlin, Germany) sequences pre-processed with Geneious® 9.1.8 (Biomatters, Auckland, New Zealand) and compared to sequences from the GenBank database (National Center for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Kruskal-Wallis tests were used to test for significant differences in the number of mosquitoes between the two overwintering site types (animal burrow vs. anthropogenic site). The test was applied separately for *Cx. p. pipiens* and *Cx. torrentium*. Statistical analysis and visualisation of the results were conducted with the R version 4.2.0 [33], including the package ggplot2, version 3.4.0 [34].

3. Results

The retrospective molecular typing of the *Cx. pipiens* s.s./*Cx. torrentium* specimens from the entomological study on burrows by Myczkoet al. [27] revealed a dominance of >60% for *Cx. torrentium* (Table 1). During the systematic study in 2022, mosquitoes were found in 15 of the 20 sampled animal burrows and 22 of the 23 sampled anthropogenic overwintering sites. In total, 4710 female mosquitoes were collected from the anthropogenic overwintering sites (maximum per site: 2446 specimens) and 1688 from the animal burrows (maximum per site: 739 specimens) (Supplementary Table S1). In the anthropogenic overwintering sites, this included 57 *Anopheles maculipennis* s.l. Meigen, 1818 (1%), 639 *Culiseta annulata/subochrea* (14%), 31 *Culex territans/hortensis* Walker, 1856 (1%), 3378 *Cx. p. pipiens* (72%) and 47 *Cx. torrentium* (1%) (Table 1). 552 *Cx. pipiens* s.s./*Cx. torrentium* (12%) could not be clearly identified since the mosquito pools were positive for *Cx. p. pipiens* and *Cx. torrentium*. These pools were positive for both *Cx. pipiens* s.s. and *Cx. torrentium* species in the real-time PCR assay. In the animal burrows, 1688 *Cx. pipiens* s.s./*Cx torrentium* were collected, of which 129 (8%) were typed as *Cx. p. pipiens* and 880 (52%) as *Cx. torrentium*. Pools with a total of 679 specimens (40%) were positive for both, *Cx. p. pipiens* and *Cx. torrentium*. The number of *Cx. p. pipiens* per site was significantly higher at anthropogenic overwintering sites than in animal burrows (Chi-Squared = 8.4, df = 1, $p = 0.004$), whereas the number of *Cx. torrentium* per site was significantly higher in animal burrows compared to anthropogenic overwintering sites (Chi-squared =

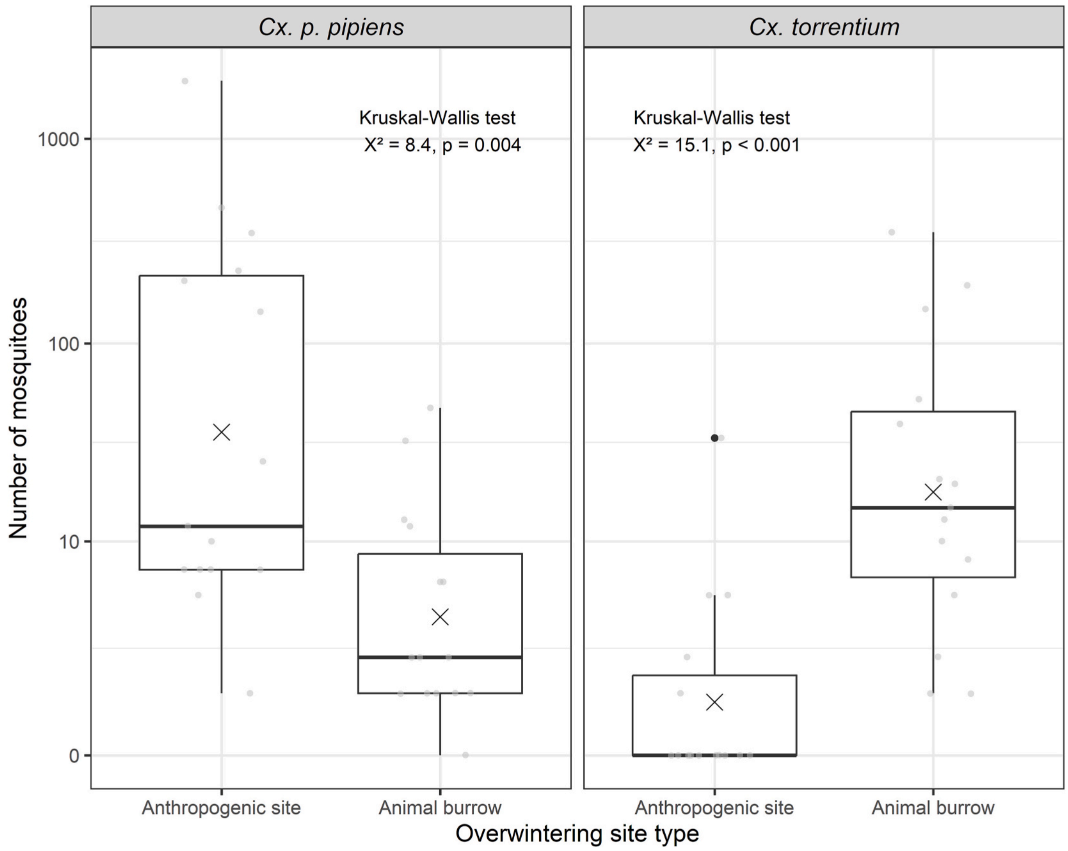

Fig. 1. Boxplots of the number of *Cx. p. pipiens* and *Cx. torrentium* specimens per overwintering site type. The "x" indiactes the mean.

15.1, df = 1, $p < 0.001$) (Fig. 1). In both years, most mosquitoes left the animal burrows between the end of March and the end of April, peaking in the middle of April (Fig. 2). No further mosquitoes were collected from the animal burrows after May 10, 2022. A single pool of ten *Cx. torrentium* specimens collected on April 14, 2022 from an animal burrow (52°27′49.7"N 16°51′33.2″E) was positive for Usutu virus (USUV).

4. Discussion

The systematic comparison of two types of overwintering sites demonstrated that high numbers of *Cx. torrentium* overwinter in animal burrows, whereas the species is underrepresented in anthropogenic overwintering sites (cellars and abandoned aboveground buildings). The opposite pattern was observed for *Cx. p. pipiens*. The dominance of *Cx. p. pipiens* in anthropogenic overwintering sites is in line with previous studies in Europe [22–24]. Nevertheless, overwintering *Cx. p. pipiens* and *Cx. torrentium* have been found together in similar numbers in rock caves and mine shafts [35,36]. In our study, natural rock caves were not included as these structures are extremely rare in the area. This is the case in many landscapes, especially lowlands or urban areas. Hence, other natural structures must play an important role as overwintering sites for mosquitoes in landscapes without caves. Studies from North America, where *Cx. torrentium* is absent, have shown that *Cx. tarsalis* Coquillett, 1896 overwinters in animal burrows [37,38]. In this study, we report high numbers of *Cx. torrentium* in animal burrows. Additionally, based on the comparison of specimens identified to species level, *Cx. torrentium* was much more abundant in animal burrows compared with *Cx. p. pipiens*. In the light of the distinct overwintering site pattern, animal burrows may be the primary overwintering site for *Cx. torrentium*, potentially distinguishing this species ecologically from *Cx. p. pipiens*. Although *Cx. p. pipiens* and *Cx. torrentium* are known to differ in their vector capacity, e.g. vector competence [4,5] or *Wolbachia* infection [39,40], their ecological differences are unclear, e.g. breeding ecology [41] or host-feeding patterns [42]. Following the concept of limiting similarity in community ecology proposed by MacArthur and Levins [43], two species with the same ecological niche are not be able to coexist. The different overwintering site patterns of *Cx. p. pipiens* and *Cx. torrentium* might represent one ecological niche difference and can possibly help to further explain the sympatric coexistence of the two species in Central Europe. Remarkably, only *Cx. p. pipiens* and *Cx. torrentium* were collected from the animal burrows, although different other mosquito taxa were found in nearby anthropogenic sites, including *An. maculipennis* s.l., *Cx. territans/hortensis* and *Cs. annulata/subochrea*. The natural overwintering sites of these adult overwintering mosquito taxa are unclear. Thus, there is a need for further investigation of the natural overwintering sites of mosquitoes, including the wide variety of burrow types dug by different vertebrate species. The lack of knowledge also applies to environmental cues associated with the selection of overwintering sites by mosquitoes in general.

Previous serological studies from Poland demonstrated WNV- and USUV-specific antibodies in birds, horses and humans, including in the Greater Poland area, where our study was conducted [44,45]. To our knowledge, the USUV-positive pool in our study represents the first detection of USUV RNA in Poland, thereby underscoring the ongoing virus circulation in Poland. Previous studies on arboviruses in overwintering mosquitoes in Europe detected WNV and SINV in *Cx. p. pipiens* [17,23] as well as WNV and Ťahyňa virus in undifferentiated *Cx. pipiens* s.s./*Cx. torrentium* specimens [15,46]. Thus, the USUV detection in our study provide the first evidence, that *Cx. torrentium* can act as a winter reservoir for arboviruses. Considering the vector competence of *Cx. torrentium* for SINV, WNV and USUV [4–7], infected females are very likely capable to transmit these viruses in the following spring and thus contribute to the arbovirus persistence in Central Europe. However, we detected only a single positive pool of ten specimens, which corresponds

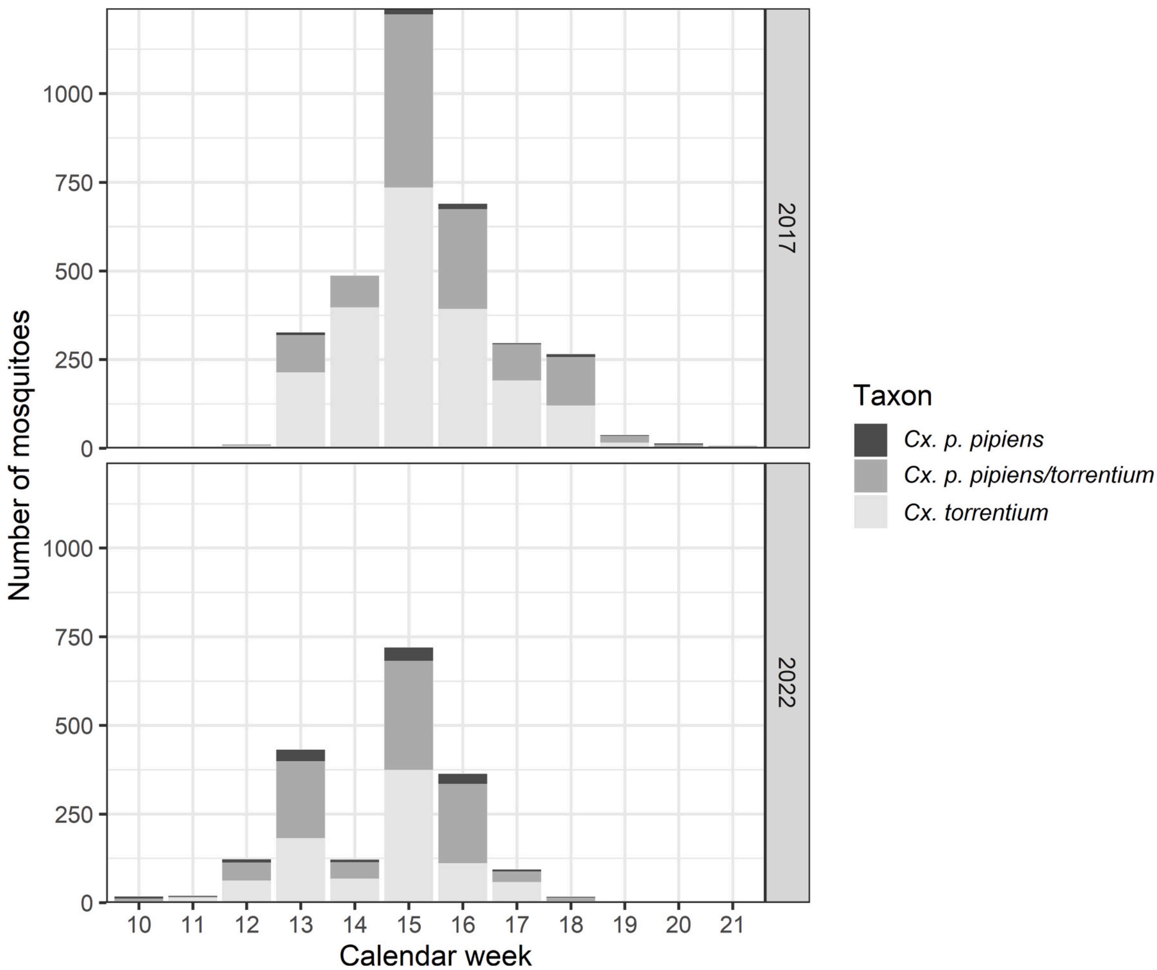

Fig. 2. Total number of *Cx. p. pipiens* and *Cx. torrentium* specimens collected from the animal burrows in 2017 (Myczko et al. 2021) and in 2022.

Table 1
Total number of mosquitoes analysed from the study in 2017 (Myczko et al. 2021) and collected in the field study in 2022.

	2017	2022	
Mosquito taxon	Animal burrows	Animal burrows	Anthropogenic sites
Anopheles maculipennis s.l.	0	0	57
Culex territans/hortensis	0	0	31
Culex p. pipiens	55	129	3378
Culex torrentium	2077	880	47
Culex p. pipiens/Cx. torrentium	1323	679	552
Culiseta annulata/ subochrea	0	0	639
Culiseta spp.	0	0	6

to a minimum infection rate of 0.18% for all analysed *Cx. pipiens* s.s./*Cx. torrentium* specimens from 2022. This low infection rate is similar to studies that screened overwintering *Culex* for WNV in the United States [12–14], the Czech Republic [15], Russia [16] or Germany [17]. In addition, no virus-positive mosquitoes were found in a study from the Netherlands [47] and Italy [48], even though WNV and USUV had circulated in the preceding summers in the study areas. Hence, the authors suggest that other mechanisms might be important for arbovirus overwintering in Europe [47,48].

Reisen and Wheeler [49] reviewed three possible pathways of WNV overwintering in US: regular reintroduction, persistence in vertebrate hosts or persistence in mosquitoes. The circulation of USUV and WNV in Europe is driven by complex interplay of dispersal, local establishment and evolution [50]. However, the ongoing circulation and dominance of the same distinct arbovirus subclades indicate successful overwintering of arboviruses, instead of regular introduction [51,52]. Long-term infections, recrudescence or bird-to-bird transmission is probably possible for both viruses [49,53]. It is very likely that different overwintering mechanisms play a certain role for the long-term, local establishment of arboviruses. Due to the different vector capacity within the European *Cx. pipiens* s.s./*Cx. torrentium* [4–6], it is especially important to genetically differentiate the taxa in future studies on overwintering mosquitoes. Moreover, the detection rates in mosquitoes might be biased, because the field-sampled mosquitoes are usually directly frozen. Dohm and Turell [54] demonstrated that the living mosquitoes should be kept several days at temperatures higher than 20 °C for efficient arbovirus replication within the vectors, and thus increasing the detection sensitivity.

In conclusion, *Cx. torrentium* overwinters in abandoned animal burrows and can act as winter reservoir for arboviruses such as USUV.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2023.100572.

Contributions

Designed the study: Felix G. Sauer, Łukasz Myczko, Renke Lühken.
Data collection: Felix G. Sauer, Łukasz Myczko, Unchana Lange, Blanka Wiatrowska.
Data analysis: Felix G. Sauer, Łukasz Myczko, Renke Lühken.
Wrote the manuscript: Felix G. Sauer, Renke Lühken.

Revised the manuscript: Łukasz Myczko, Ellen Kiel, Blanka Wiatrowska, Jonas Schmidt-Chanasit.

All authors approved the submitted version.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Data availability

Data will be made available on request.

Acknowledgement

We thank Forestry Experimental Station in Murowana Goślina, for access to the forest stands and buildings, and facilitating the fieldwork. We thank institutions and private owners for access to anthropogenic overwintering sites. We would like to acknowledge Przemysław Kurek, Aleksandra Kraśkiewicz and Jacek Więckowski for their assistance in mosquitoes capture field work. RL and FGS are funded by the Federal Ministry of Education and Research of Germany (Grant Number 01Kl2022). In addition, FGS was funded through the German Federal Ministry of Health (ZMI1-2521NIK400).

References

[1] N. Becker, D. Petrić, M. Zgomba, C. Boase, M.B. Madon, C. Dahl, A. Kaiser, in: T. Edition (Ed.), Mosquitoes Identification, Ecology and Control, Springer, 2020.

[2] D.M. Fonseca, N. Keyghobadi, C.A. Malcolm, C. Mehmet, F. Schaffner, M. Mogi, R. C. Fleischer, R.C. Wilkerson, Emerging vectors in the *Culex pipiens* Complex, Science 303 (2004) 1535–1538.

[3] Y. Haba, L. McBride, Origin and status of *Culex pipiens* mosquito ecotypes, Curr. Biol. 32 (5) (2022) R237–R246.

[4] J.C. Hesson, J. Verner-Carlsson, A. Larsson, R. Ahmed, Å. Lundkvist, J. O. Lundström, *Culex torrentium* mosquito role as major enzootic vector defined by rate of Sindbis virus infection, Sweden, 2009, Emerg. Infect. Dis. 21 (5) (2015) 875.

[5] S. Jansen, A. Heitmann, R. Lühken, M. Leggewie, M. Helms, M. Badusche, G. Rossini, J. Schmidt-Chanasit, E. Tannich, *Culex torrentium*: a potent vector for the transmission of West Nile virus in Central Europe, Viruses 11 (6) (2019) 492.

[6] S. Jansen, A. Heitmann, R. Uusitalo, E.M. Korhonen, R. Lühken, K. Kliemke, U. Lange, M. Helms, L. Kirjalainen, R. Nykänen, H. Gregow, P. Pirinen, G. Rossini, O. Vapalahti, J. Schmidt-Chanasit, E. Huhtamo, Vector competence of northern European *Culex pipiens* biotype *pipiens* and *Culex torrentium* to West Nile and Sindbis viruses, Viruses 15 (3) (2023) 592.

[7] C.M. Holicki, D.E. Scheuch, U. Ziegler, J. Lettow, H. Kampen, D. Werner, M. H. Groschup, German *Culex pipiens* biotype *molestus* and *Culex torrentium* are vector-competent for Usutu virus, Parasit. Vectors 13 (1) (2020) 625.

[8] W.K. Reisen, S.S. Wheeler, Overwintering of West Nile virus in the United States, J. Med. Entomol. 56 (6) (2019) 1498–1507.

[9] D.L. Denlinger, P.A. Armbruster, Mosquito diapause, Annu. Rev. Entomol. 59 (2014) 73–93.

[10] L.B. Goddard, A.E. Roth, W.K. Reisen, T.W. Scott, Vertical transmission of west nile virus by three California *Culex* (Diptera: Culicidae) species, J. Med. Entomol. 40 (6) (2003) 743–746.

[11] D.J. Dohm, M.R. Sardelis, M.J. Turell, Experimental vertical transmission of West Nile virus by *Culex pipiens* (Diptera: Culicidae), J. Med. Entomol. 39 (4) (2002) 640–644.

[12] R.S. Nasci, H.M. Savage, D.J. White, J.R. Miller, B.C. Cropp, M.S. Godsey, A. J. Kerst, P. Bennett, K. Gottfried, R.S. Lanciotti, West Nile virus in overwintering *Culex* mosquitoes, new York City, 2000, Emerg. Infect. Dis. 7 (4) (2001) 742–744.

[13] L.M. Bugbee, L.R. Forte, The discovery of West Nile virus in overwintering *Culex pipiens* (Diptera: Culicidae) mosquitoes in Lehigh County, Pennsylvania, J. Am. Mosq. Control Assoc. 20 (3) (2004) 326–327.

[14] A. Farajollahi, W.J. Crans, P. Bryant, B. Wolf, K.L. Burkhalter, M.S. Godsey, S. E. Aspen, R.S. Nasci, Detection of West Nile viral RNA from an overwintering pool of *Culex pipens pipiens* (Diptera: Culicidae) in New Jersey, 2003, J. Med. Entomol. 42 (3) (2005) 490–494.

[15] I. Rudolf, L. Betášová, H. Blažejová, K. Venclíková, P. Straková, O. Šebesta, J. Mendel, T. Bakonyi, F. Schaffner, N. Nowotny, Z. Hubálek, West Nile virus in overwintering mosquitoes, Central Europe, Parasit. Vectors 10 (1) (2017) 452.

[16] N.V. Borodai, A.V. Nesgovorova, V.K. Fomina, A.K. Mendygalieva, A.A. Baturin, A. S. Antonov, E.F. Avdiusheva, E.V. Molchanova, D.N. Nikitin, E.V. Putintseva, Detection of West Nile virus in overwintering mosquitoes in the Volgograd region, Public Health Life Environ. 4 (2022) 70–76.

[17] H. Kampen, B.A. Tews, D. Werner, First evidence of West Nile virus overwintering in mosquitoes in Germany, Viruses 13 (12) (2021) 2463.

[18] M. Schäfer, J.O. Lundström, Comparison of mosquito (Diptera: Culicidae) Fauna characteristics of forested wetlands in Sweden, Ann. Entomol. Soc. Am. 94 (4) (2001) 576–582.

[19] S. Sulaiman, M. Service, Studies on hibernating populations of the mosquito *Culex pipiens* L. in southern and northern England, J. Nat. Hist. 17 (6) (1983) 849–857.

[20] J.D. Buffington, Hibernaculum choice in *Culex pipiens*, J. Med. Entomol. 9 (2) (1972) 128–132.

[21] P. Jupp, *Culex* (*Culex*) *pipiens* and *Culex* (*Culex*) *torrentium* (Diptera: Culicidae) in England: notes on their taxonomy and biology, Mosquito Syst. (USA) 11 (1979) 121–126.

[22] T.G. Jaenson, Overwintering of *Culex* mosquitoes in Sweden and their potential as reservoirs of human pathogens, Med. Vet. Entomol. 1 (2) (1987) 151–156.

[23] A. Bergman, E. Dahl, Å. Lundkvist, J.C. Hesson, Sindbis virus infection in non-blood-fed hibernating *Culex pipiens* mosquitoes in Sweden, Viruses 12 (12) (2020) 1441.

[24] F.G. Sauer, E. Timmermann, U. Lange, R. Lühken, E. Kiel, Effects of hibernation site, temperature, and humidity on the abundance and survival of overwintering *Culex pipiens pipiens* and *Anopheles messeae* (Diptera: Culicidae), J. Med. Entomol. 59 (6) (2022) 2013–2021.

[25] J. Börstler, R. Lühken, M. Rudolf, S. Steinke, C. Melaun, S. Becker, R. Garms, A. Krüger, The use of morphometric wing characters to discriminate female *Culex pipiens* and *Culex torrentium*, J. Vect. Ecol. 39 (1) (2014) 204–212.

[26] O.W. Lwande, J. Näslund, E. Lundmark, K. Ahlm, C. Ahlm, G. Bucht, M. Evander, Experimental infection and transmission competence of Sindbis virus in *Culex torrentium* and *Culex pipiens* mosquitoes from northern Sweden, Vector-Borne Zoonot. Dis. 19 (2) (2019) 128–133.

[27] Ł. Myczko, P. Kurek, P. Tryjanowski, B. Wiatrowska, Ł. Jankowiak, Ł. Mielczarek, P. Sienkiewicz, T. Rutkowski, A. Ondrejková, Where to overwinter: burrows of medium-sized carnivores as winter places for invertebrates in temperate environment, Ecol. Entomol. 46 (5) (2021) 1177–1184.

[28] R. Kowalczyk, B. Jędrzejewska, A. Zalewski, W. Jędrzejewski, Facilitative interactions between the Eurasian badger (*Meles meles*), the red fox (*Vulpes vulpes*), and the invasive raccoon dog (*Nyctereutes procyonoides*) in Białowieża primeval Forest, Poland, Can. J. Zool. 86 (12) (2008) 1389–1396.

[29] K. Nowakowski, A. Ważna, P. Kurek, J. Cichocki, G. Gabryś, Reproduction success in European badgers, red foxes and raccoon dogs in relation to sett cohabitation, PLoS One 15 (8) (2020), e0237642.

[30] G.M. Vazquez-Prokopec, W.A. Galvin, R. Kelly, U. Kitron, A new, cost-effective, battery-powered aspirator for adult mosquito collections, J. Med. Entomol. 46 (6) (2009) 1256–1259.

[31] M. Rudolf, C. Czajka, J. Börstler, C. Melaun, H. Jöst, H. von Thien, M. Badusche, N. Becker, J. Schmidt-Chanasit, A. Krüger, First nationwide surveillance of *Culex pipiens* complex and *Culex torrentium* mosquitoes demonstrated the presence of *Culex pipiens* biotype *pipiens/molestus* hybrids in Germany, PLoS One 8 (9) (2013), e71832.

[32] D.-Y. Chao, B.S. Davis, G.-J.J. Chang, Development of multiplex real-time reverse transcriptase PCR assays for detecting eight medically important flaviviruses in mosquitoes, J. Clin. Microbiol. 45 (2) (2007) 584–589.

[33] R Core Team, R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2022.

[34] H. Wickham, ggplot2: Elegant Graphics for Data Analysis, Springer-Verlag, New York, 2016.

[35] D.D. Dörge, S. Cunze, H. Schleifenbaum, S. Zaenker, S. Klimpel, An investigation of hibernating members from the *Culex pipiens* complex (Diptera, culicidae) in subterranean habitats of Central Germany, Sci. Rep. 10 (1) (2020) 1–11.

[36] C. Zittra, S. Vitecek, J. Teixeira, D. Weber, B. Schindelegger, F. Schaffner, A. M. Weigand, Mosquitoes (Diptera: Culicidae) in the dark—highlighting the importance of genetically identifying mosquito populations in subterranean environments of Central Europe, Pathogens 10 (9) (2021) 1090.

[37] R. Harwood, Trapping overwintering adults of the mosquitoes *Culex tarsalis* and *Anopheles freeborni*, Mosquito News 22 (1) (1962).

[38] J. Shemanchuk, On the hibernation of *Culex tarsalis* Coquillett, *Culiseta inornata* Williston, and *Anopheles earlei* Vargas, (Diptera: Culicidae) in Alberta, Mosquito Nevos 25 (4) (1965).

[39] A. Bergman, J.C. Hesson, Wolbachia prevalence in the vector species *Culex pipiens* and *Culex torrentium in* a Sindbis virus-endemic region of Sweden, Parasit. Vectors 14 (1) (2021) 1–7.

[40] M. Leggewie, R. Krumkamp, M. Badusche, A. Heitmann, S. Jansen, J. Schmidt-Chanasit, E. Tannich, S. Becker, *Culex torrentium* mosquitoes from Germany are negative for Wolbachia, Med. Vet. Entomol. 32 (1) (2018) 115–120.

[41] R. Lühken, S. Steinke, M. Leggewie, E. Tannich, A. Krüger, S. Becker, E. Kiel, Physico-chemical characteristics of *Culex pipiens* sensu lato and Culex torrentium (Diptera: Culicidae) breeding sites in Germany, J. Med. Entomol. 52 (5) (2015) 932–936.

[42] J. Börstler, H. Jost, R. Garms, A. Kruger, E. Tannich, N. Becker, J. Schmidt-Chanasit, R. Lühken, Host-feeding patterns of mosquito species in Germany, Parasit. Vectors 9 (1) (2016) 318.

[43] R. MacArthur, R. Levins, The limiting similarity, convergence, and divergence of coexisting species, Am. Nat. 101 (921) (1967) 377–385.

[44] B. Bażanów, P. Jansen van Vuren, P. Szymański, D. Stygar, A. Frącka, J. Twardoń, R. Kozdrowski, J.T. Pawęska, A survey on West Nile and Usutu viruses in horses and birds in Poland, Viruses 10 (2) (2018) 87.

[45] J.S. Niczyporuk, E. Samorek-Salamonowicz, S. Lecollinet, S.A. Pancewicz, W. Kozdruń, H. Czekaj, Occurrence of West Nile virus antibodies in wild birds, horses, and humans in Poland, Biomed. Res. Int. 2015 (2015), 234181.

[46] I. Rudolf, S. Šikutová, O. Šebesta, J. Mendel, I. Malenovský, H. Kampen, J. Medlock, F. Schaffner, Overwintering of *Culex modestus* and other mosquito species in a reedbed ecosystem, including arbovirus findings, J. Am. Mosq. Control Assoc. 36 (4) (2020) 257–260.
[47] R. Blom, M.J. Schrama, J. Spitzen, B.F. Weller, A. van der Linden, R.S. Sikkema, M. P. Koopmans, C.J. Koenraadt, Arbovirus persistence in North-Western Europe: are mosquitoes the only overwintering pathway? One Health 16 (2023), 100467.
[48] M. Calzolari, P. Bonilauri, R. Bellini, A. Albieri, F. Defilippo, M. Tamba, M. Tassinari, A. Gelati, P. Cordioli, P. Angelini, Usutu virus persistence and West Nile virus inactivity in the Emilia-Romagna region (Italy) in 2011, PLoS One 8 (5) (2013), e63978.
[49] W.K. Reisen, S.S. Wheeler, Overwintering of West Nile virus in the United States, J. Med. Entomol. 56 (6) (2019) 1498–1507.
[50] D. Engel, H. Jöst, M. Wink, J. Börstler, S. Bosch, M.-M. Garigliany, A. Jöst, C. Czajka, R. Lühken, U. Ziegler, Reconstruction of the evolutionary history and dispersal of Usutu virus, a neglected emerging arbovirus in Europe and Africa, MBio 7 (1) (2016) e01938-15.
[51] U. Ziegler, P.D. Santos, M.H. Groschup, C. Hattendorf, M. Eiden, D. Höper, P. Eisermann, M. Keller, F. Michel, R. Klopfleisch, K. Müller, D. Werner, H. Kampen, M. Beer, C. Frank, L. Lachmann, T. Grünewald, C.A. Szentiks, M. Sieg, J. Schmidt-Chanasit, D. Cadar, R. Lühken, West Nile virus epidemic in Germany triggered by epizootic emergence, 2019, Viruses 12 (4) (2020) 448.
[52] G. Mencattelli, F. Iapaolo, A. Polci, M. Marcacci, A. Di Gennaro, L. Teodori, V. Curini, V. Di Lollo, B. Secondini, S. Scialabba, West Nile virus lineage 2 overwintering in Italy, Trop. Med. Infect. Dis. 7 (8) (2022) 160.
[53] V. Schmidt, K. Cramer, D. Böttcher, K. Heenemann, A. Rückner, M. Harzer, U. Ziegler, T. Vahlenkamp, M. Sieg, Usutu virus infection in aviary birds during the cold season, Avian Pathol. 50 (5) (2021) 427–435.
[54] D.J. Dohm, M.J. Turell, Effect of incubation at overwintering temperatures on the replication of West Nile virus in New York Culex pipiens (Diptera: Culicidae), J. Med. Entomol. 38 (3) (2001) 462–464.