



First record of the West Nile virus bridge vector *Culex modestus* Ficalbi (Diptera: Culicidae) in Belgium, validated by DNA barcoding

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Abstract

A thorough knowledge of the presence and spatio-temporal distribution patterns of vector species are pivotal to assess the risk of mosquito-borne diseases in Europe. In 2018, a *Culex* larva was collected during routine monitoring activities to intercept exotic *Aedes* mosquito species in the port of Antwerp (Kallo, Belgium). The larva, collected from a pond in mid-September, was morphologically identified as *Culex modestus*, and this identification was subsequently confirmed by *COI* barcoding. It is the first confirmed record of this West Nile virus bridge vector in Belgium. The present study also demonstrates the value of DNA-based identification techniques to validate the presence of potential vector species.

Key words: biodiversity, DNA-based identification, larva, monitoring, mosquito, new record

Introduction

Mosquitoes are notorious for their ability to transmit several pathogens of human diseases, and high costs are usually associated with the implementation of prevention and control strategies, health care, loss of economic productivity or recreational activities (Roiz *et al.* 2018; Thompson *et al.* 2020). Main factors contributing to the (re-)emergence of diseases caused by mosquito-borne pathogens are increased globalisation (causing introduction of exotic vectors and/or pathogens), altered landscape management (e.g. wetland restoration, urbanisation), changing socioeconomic behaviour and climatic changes (Becker 2008; Randolph & Rogers 2010; Roiz *et al.* 2015; Rochlin *et al.* 2016). In this context, thorough knowledge of mosquito diversity is crucial to assess the current and future risks of the local transmission of pathogens (Versteirt *et al.* 2013; Medlock *et al.* 2015; Calzolari 2016). For example, local chikungunya and dengue fever outbreaks in southern Europe resulted from local transmission of travel-related arboviruses by the established exotic vector species *Aedes albopictus* (Skuse) (ECDC 2018). Also, several indigenous European mosquito species can transmit mosquito-borne pathogens, such as West Nile, Tahyna, Sindbis, Batai and Inkoo viruses (Lundström 1999; Hubálek 2008).

In Europe, West Nile virus (WNV) has been showing a strong resurgence since 1996 (Hubálek & Halouzka 1999; Zeller & Schuffenecker 2004). From 2010 onwards, WNV has engendered a permanent public health problem in Europe (Papa *et al.* 2010; Zeller *et al.* 2010; Ziegler *et al.* 2019). Its suspected or proven main vectors in this region are *Culex pipiens* Linnaeus, *Cx. modestus* Ficalbi and *Cx. perexiguus* Theobald (Balenghien *et al.* 2006; Balenghien *et al.* 2007; Becker *et al.* 2010; Brugman *et al.* 2018). These three species regularly feed on birds, humans and other mammals (Becker *et al.* 2010; Radrova *et al.* 2013; Brugman *et al.* 2017). As such, *Cx. modestus* is the principal WNV bridge vector in the Camargue wetlands of France (Ponçon *et al.* 2007). While *Cx. pipiens* is a widespread and abundant species in Belgium, *Cx. modestus* has not yet been recorded in the country (Versteirt *et al.* 2013; Boukraa *et al.* 2015). This study presents the first morphological and molecular evidence of its occurrence in Belgium.

Material and methods

Larval sampling

Within the framework of the ‘Monitoring of Exotic MOSquito species’ (MEMO) project, mosquito larvae were collected from April until November 2017, 2018 and 2019 at 23 localities in Belgium (Deblauwe *et al.* 2020). Larval sampling targeted predominantly artificial container habitats (e.g. flower vases, rain barrels, tyres), as these are the preferred larval habitats of exotic *Aedes* species, but also some natural sites were sporadically sampled. Fine-meshed aquarium nets were used to collect larvae from the water. The larvae were killed by a heat shock at 70°C in the laboratory, and subsequently transferred in 80% ethanol for storage at room temperature before species identification.

Morphological and DNA-based species identification

Larvae were morphologically identified using the keys of Becker *et al.* (2010) and Gunay *et al.* (2018). For every species, one or multiple larvae were selected as voucher specimens. After morphological identification, the anterior part of each larval abdomen was used for DNA-based species identification, the head and posterior part of the abdomen of these voucher larvae were slide-mounted using dimethylhydantoin formaldehyde resin, microscope cavity slides and square cover slips. The voucher specimens and their respective DNA were submitted to the Belgian Culicidae collection housed in the Royal Belgian Institute of Natural Sciences.

DNA-based species identification was performed to confirm the morphological species identifications. Therefore, DNA was extracted from the anterior part of the larval abdomen using the NucleoSpin® Tissue extraction kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) following the manufacturer’s protocol, but with the elution volume set to 70 µl. A fragment of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene was amplified using the universal primers LCO1490 and HCO2198 (Folmer *et al.* 1994). PCR reactions and conditions, purification and sequencing were carried out as done by Ibáñez-Justicia *et al.* (2020). Finally, the *COI* consensus sequence was compared against the BLAST web application of GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and compared against the Identification System of BOLD, with the Species Level Barcode Records option (www.boldsystems.org).

To proceed with the species validation of the suspected *Cx. modestus* larva, all publicly available *COI* sequences of *Culex* species known to occur in Belgium (Boukraa *et al.* 2015), and all the *COI* sequences of *Cx. modestus*, were downloaded from the BOLD online repository (Table 1; <http://www.boldsystems.org/index.php/databases>; November 2019). Sequences with a minimum size of 300 bp were retained, aligned using ClustalW in Geneious® v10.0.4 (Biomatters Ltd, Auckland, New Zealand) and checked for stop codons. *COI* sequences of *Coquillettia richiardii* (Ficalbi) (KM25803, KU876993) and *Cq. fuscopennata* (Theobald) (GQ165802) were included as out-group, and the alignment was then trimmed to only retain the 658-bp *COI* region (Folmer *et al.* 1994). Duplicate sequences were discarded per species using BioEdit v.7.2.6 (Hall 1999). Subsequently, a rooted Neighbor-Joining (NJ) tree based on Tamura-Nei distances was constructed using Geneious® v10.0.4. Nodal support was assessed by bootstrapping over 1000 replicates with a cut-off threshold of 70%.

TABLE 1. List of *Culex* species found to occur in Belgium (Boukraa *et al.* 2015), including the newly recorded *Cx. modestus*, their general occurrence in Belgium and the number of *COI* sequences (N_{COI}) downloaded from the BOLD repository (November 2019). The number of unique sequences (as defined by BioEdit) are enclosed in parentheses. Sequences were downloaded for neighbouring countries with confirmed occurrences of *Cx. modestus*. The alignment was delimited to the barcode region (Folmer *et al.* 1994).

Species	Occurrence in Belgium	Total N_{COI}	N_{COI} Belgium
<i>Cx. territans</i>	Rare	260 (228)	4 (4)
<i>Cx. pipiens</i>	Common	945 (213)	34 (10)
<i>Cx. hortensis</i>	Rare	18 (17)	0
<i>Cx. torrentium</i>	Common	340 (108)	5 (5)
<i>Cx. modestus</i>	First record	247 (121)	0

TABLE 1. (Continued)

Species	N_{COI} neighbouring countries				
	France	Luxembourg	Germany	Netherlands	United Kingdom
<i>Cx. territans</i>	0	0	1	0	0
<i>Cx. pipiens</i>	0	0	424 (13)	0	146 (16)
<i>Cx. hortensis</i>	1	0	15 (14)	0	0
<i>Cx. torrentium</i>	0	0	282 (26)	0	36 (22)
<i>Cx. modestus</i>	30 (18)	0	51 (26)	0	98 (27)

Results

Over the entire study period (2017–2019), one larva was morphologically identified as *Cx. modestus*. The larva was distinguished from other *Culex* species by the absence of lateral siphonal setae, the absence of a distinct median spine on the comb scales, the siphonal index (total length / diameter at base) between 3 and 4.5 (here 4.5) and the straight sides of the siphon. Most typical are the ventral siphonal setae, which appear in disarray (Fig. 1A), in contrast to the paired siphonal setae of the commonly collected and closely related species *Cx. pipiens* (Fig. 1B) and *Cx. torrentium* Martini (Becker *et al.* 2010; Harbach 2012).

The larva was collected on 18 September 2018 in a small pond close to the port of Antwerp (Kallo) in the province of East Flanders (51° 15' 11.14" N, 4° 12' 48.53" E). The vernal pond was mainly vegetated with common cattail (*Typha latifolia*), and was almost completely dried out at the time of the sampling (Fig. 2). In addition, larvae of *Culiseta annulata* Schrank ($n = 1$), *Anopheles maculipennis* Meigen *s.l.* ($n = 1$) and *Cx. pipiens* ($n = 4$) were collected at the same place on the same day. On 13 November 2018, the same pond was sampled again, but no mosquito larvae were found.

After sequence blasting in BOLD and GenBank, a 100% similarity was found with *COI* sequences of *Cx. modestus* from the United Kingdom (BOLD record CXOMD049-17, GenBank accession MK971827). The next 100 best matches all involved *Cx. modestus* *COI* sequences. The species identification was validated by the NJ tree, in which the generated consensus *COI* sequence of the Belgian specimen clustered with high confidence (81.6% bootstrap value) with other *Cx. modestus* *COI* sequences (Fig. 3). The *COI* barcode of the specimen was deposited in GenBank (accession MN978924). Thereby, DNA-based species identification confirmed the morphological identification of the larva.

Discussion

This is the first report of *Cx. modestus* in Belgium, based on the single larva collected at Kallo. The identification of the larva was confirmed by both morphology and *COI* barcoding. However, the species was expected to occur in Belgium based on its presence in neighbouring countries, and some suspected larvae collected at two cattle farms in Brecht and Somme-Leuze (Belgium) during 2008 and 2009 (Table 1) (Boukraa *et al.* 2011; Ries 2019; ECDC

2020). Nevertheless, in the absence of well-confirmed records it was not included in the latest checklist of the mosquito species known to occur in Belgium (Boukraa *et al.* 2015). Morphological identification of immature life stages, damaged specimens and sibling species of species complexes can quickly become challenging (Hebert *et al.* 2003; Versteirt *et al.* 2015). In these situations, DNA barcoding can support monitoring campaigns and help at providing correct species identifications.

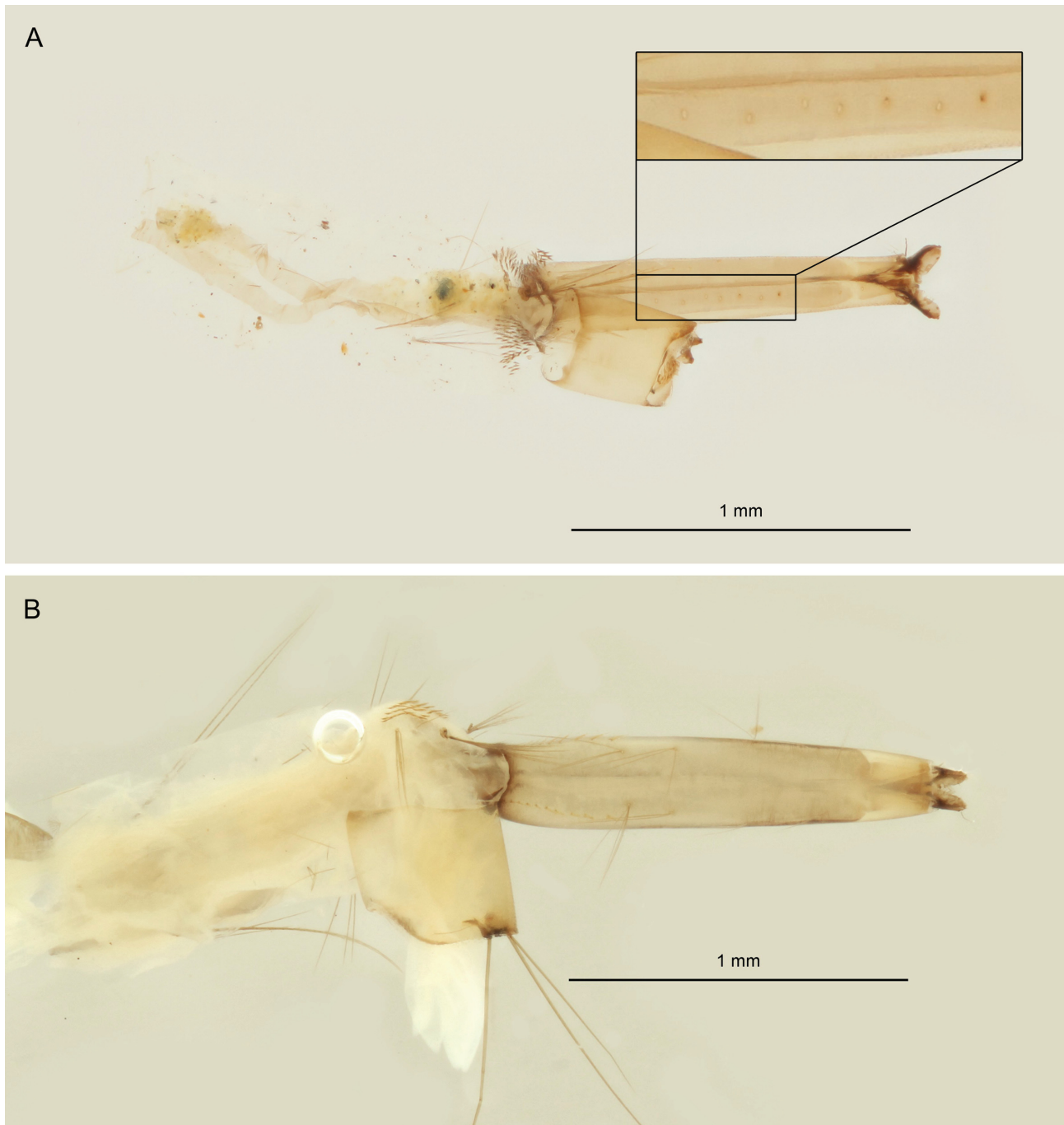


FIGURE 1. (A) Posterior part of the mounted *Cx. modestus* larva. Zoom on the diagnostic characteristic of the siphon, showing disarrayed insertion points of the ventral siphonal setae. (B) Posterior part of a mounted *Cx. pipiens* larva.

The larva was collected in a small pond in the port area of Antwerp, which is part of a protected ecological corridor network constructed in 2016. This network consists of several aquatic sites preserved for the reproduction of endangered freshwater species, e.g. the Natterjack toad *Epidalea calamita* Laurenti. The port area is rich in freshwater to slightly saline wetlands, ponds and marshes, which are the preferred habitats of *Cx. modestus*, and harbour migratory and resident bird populations (Becker *et al.* 2010; Jacobs *et al.* 2020). The larvae of *Cx. modestus* show

a preference for sunlit habitats, generally characterised by rich vegetation such as reed beds, e.g. the Camargue wetlands of France (Ponçon *et al.* 2007; Radrova *et al.* 2013).

Culex modestus was not recorded during the MODIRISK project (2007–2010), a large nationwide survey conducted to inventory and assess the distribution of mosquito species (adults) in Belgium (Versteirt *et al.* 2013). Nor were larvae of this species collected during a study conducted from 1997 until 2009 to map the distribution of mosquito larvae in Flanders (Lock *et al.* 2012). However, the latter survey focused on monitoring the water quality of streams. Therefore, as stagnant waterbodies, including marshes, ponds and wetlands were underrepresented, *Cx. modestus* may have been missed. Still, while a high mosquito nuisance was reported in the port area of Kallo in 2013, no adults of *Cx. modestus* were trapped in 2014 (Sohier & Grootaert 2015).

During the MEMO monitoring project, vegetated ponds and other bodies of ground water were not among the habitats targeted for larvae of exotic *Aedes* mosquito species. So, more targeted surveys are needed to determine the actual occurrence and distribution of *Cx. modestus* in the country. For example, the species was only discovered by focused sampling of marsh areas in England (Golding *et al.* 2012). This observation is important in light of the northward spread of WNV in Europe, which led to the detection of human cases in the Netherlands in 2020 (Vlas-kamp *et al.* 2020). The increasing restoration and protection of wetlands near densely populated urban areas could create suitable habitats for potential vector species, including *Cx. modestus*, or support changes in the spatio-temporal distribution of species (Reusken *et al.* 2010; Medlock & Vaux 2015). The confirmed presence of *Cx. modestus* in Belgium confirms the trend of increased detections throughout Europe. In countries like England and the Czech Republic, studies have provided evidence of increased local abundance and spread of this previously seemingly rather rare or non-indigenous species (Radrova *et al.* 2013; Hernández-Triana *et al.* 2020).



FIGURE 2. The small vernal pond vegetated with common cattail (*Typha latifolia*) where the *Cx. modestus* larva was collected.

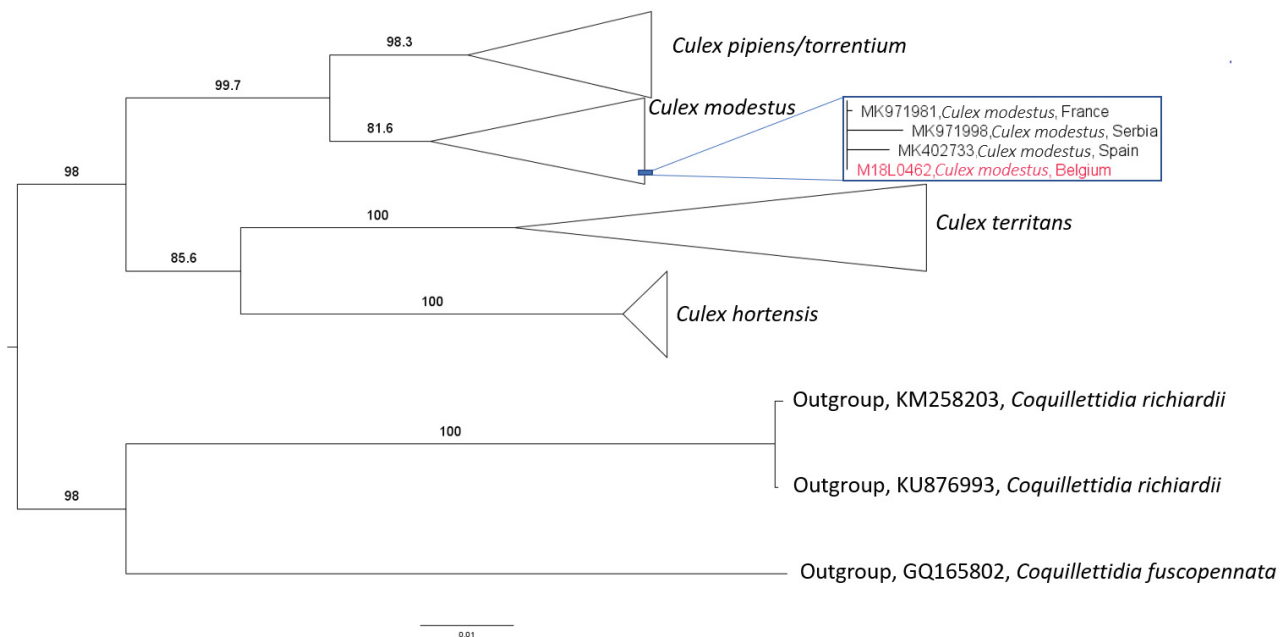


FIGURE 3. NJ tree based on *COI* sequences downloaded from BOLD, including *Culex* species recorded from Belgium (Boukraa *et al.* 2015) and sequences of *Cx. modestus* (Table 1). Bootstrap values are indicated above the branches. The blue square gives a zoom view of the un-collapsed tree.

Acknowledgements

We would like to thank all cooperating companies for giving access to their private property during mosquito sampling surveys. We want to thank the laboratory staff and technicians at the Institute of Tropical Medicine Antwerp for conducting the field work, especially Jacobus De Witte, Anna Schneider and Ingrid Verlé. We thank Slimane Boukraa (Unit of Entomology, Gembloux Agro-Bio Tech, ULiège), two anonymous reviewers and the editor for their input and improvements to the manuscript.

Funding

This work is part of the MEMO project (CES-2016-02 Belgium), funded by the Flemish, Wallonian and Brussels authorities and the FPS Health, Food Chain Safety and Environment within the framework of the Belgian national collaboration agreement in the domains of environment and health (NEHAP). The Outbreak Research Team of the Institute of Tropical Medicine is funded by the Department of Economy, Science and Innovation of the Flemish government. The Barcoding Facility for Organisms and Tissues of Policy Concern (BopCo) is part of the Belgian federal contribution of the Belgian Science Policy Office (Belspo) to the European Research Infrastructure Consortium LifeWatch.

References cited

- Balenghien, T., Fouque, F., Sabatier, P. & Bicout, D.J. (2006) Horse-, bird-, and human-seeking behavior and seasonal abundance of mosquitoes in a West Nile virus focus of southern France. *Journal of Medical Entomology*, 43 (5), 936–946. <https://doi.org/10.1093/jmedent/43.5.936>
- Balenghien, T., Vazeille, M., Reiter, P., Schaffner, F., Zeller, H. & Bicout, D.J. (2007) Evidence of laboratory vector competence of *Culex modestus* for West Nile virus. *Journal of the American Mosquito Control Association*, 23 (2), 233–236. [https://doi.org/10.2987/8756-971X\(2007\)23\[233:EOLVCO\]2.0.CO;2](https://doi.org/10.2987/8756-971X(2007)23[233:EOLVCO]2.0.CO;2)
- Becker, N. (2008) Influence of climate change on mosquito development and mosquito-borne diseases in Europe. *Parasitology*

- Research*, 103 (1), 19–28.
<https://doi.org/10.1007/s00436-008-1210-2>
- Becker, N., Petric, D., Zgomba, M., Boase, C., Madon, M., Dahl, C. & Kaiser, A. (2010) *Mosquitoes and their control*. 2nd Edition. Springer-Verlag, Berlin and Heidelberg, xxx + 577 pp.
- Boukraa, S., Dekoninck, W., Versteirt, V., Schaffner, F., Coosemans, M., Haubruge, E. & Francis, F. (2015) Updated checklist of the mosquitoes (Diptera: Culicidae) of Belgium. *Journal of Vector Ecology*, 40 (2), 398–407.
<https://doi.org/10.1111/jvec.12180>
- Boukraa, S., Zimmer, J.-Y., Simonon, G., Haubruge, E. & Francis, F. (2011) Stand structure of mosquitoes (Diptera Culicidae) in farms and establishment of a new potential vector of West Nile Virus for Belgium. *63th International Symposium on Crop Protection, Gent, Belgium*, 2011, 287.
- Brugman, V.A., England, M.E., Stoner, J., Tugwell, L., Harrup, L.E., Wilson, A.J., Medlock, J.M., Logan, J.G., Fooks, A.R., Mertens, P.P.C., Johnson, N. & Carpenter, S. (2017) How often do mosquitoes bite humans in southern England? A standardised summer trial at four sites reveals spatial, temporal and site-related variation in biting rates. *Parasites & Vectors*, 10 (1), 420–420.
<https://doi.org/10.1186/s13071-017-2360-9>
- Brugman, V.A., Hernandez-Triana, L.M., Medlock, J.M., Fooks, A.R., Carpenter, S. & Johnson, N. (2018) The role of *Culex pipiens* L. (Diptera: Culicidae) in virus transmission in Europe. *International Journal of Environmental Research and Public Health*, 15 (2), 389.
<https://doi.org/10.3390/ijerph15020389>
- Calzolari, M. (2016) Mosquito-borne diseases in Europe: an emerging public health threat. *Reports in Parasitology*, 5, 1–12.
<https://doi.org/10.2147/RIP.S56780>
- Deblauwe, I., De Wolf, K., Smits, N., Vanslebrouck, A., Schneider A., De Witte, J., Verlé, I., Dekoninck, W., De Meyer, M., Backeljau, T., Gombeer, S., Meganck, K., Van Bourgonie, Y.-R., Vanderheyden, A., Müller, R. & Van Bortel, W. (2020) *Monitoring of exotic mosquitoes in Belgium (MEMO): Final Report Phase 7 Part 1: MEMO results*. NEHAP, Antwerp, Belgium, 100 pp.
- ECDC (2018) *Local transmission of dengue fever in France and Spain – 2018*. European Centre for Disease Prevention and Control, Stockholm, Sweden, 13 pp.
- ECDC (2020) Mosquito maps, European Centre for Disease Prevention and Control and European Food Safety Authority, Stockholm. Available from: <https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/mosquito-maps> (accessed 26 August 2020)
- Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3 (5), 294–299.
- Golding, N., Nunn, M.A., Medlock, J.M., Purse, B.V., Vaux, A.G.C. & Schäfer, S.M. (2012) West Nile virus vector *Culex modestus* established in southern England. *Parasites & Vectors*, 5 (1), 32.
<https://doi.org/10.1186/1756-3305-5-32>
- Gunay, F., Picard, M. & Robert, V. (2018) MosKeyTool, an interactive identification key for mosquitoes of Euro-Mediterranean. Version 2.1. 2018. Available from: <https://www.medilabsecure.com/moskeytool.html> (accessed 26 August 2020)
- Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41 (41), 95–98.
- Harbach, R.E. (2012) *Culex pipiens*: Species versus species complex – taxonomic history and perspective. *Journal of the American Mosquito Control Association*, 28 (4s), 10–23.
<https://doi.org/10.2987/8756-971X-28.4.10>
- Hebert, P.D.N., Cywinska, A., Ball, S.L. & Dewaard, J.R. (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London, Series B, Biological Sciences*, 270 (1512), 313–321.
<https://doi.org/10.1098/rspb.2002.2218>
- Hernandez-Triana, L. M., Brugman, V. A., Pramual, P., Barrero, E., Nikolova, N. I., Ruiz-Arrondo, I., Kaiser, A., Kruger, A., Lumley, S., Osorio, H. C., Ignjatovic-Cupina, A., Petric, D., Laure Setier-Rio, M., Bodker, R. & Johnson, N. (2020) Genetic diversity and population structure of *Culex modestus* across Europe: does recent appearance in the United Kingdom reveal a tendency for geographical spread? *Medical and Veterinary Entomology*, 34 (1), 86–96.
<https://doi.org/10.1111/mve.12412>
- Hubálek, Z. (2008) Mosquito-borne viruses in Europe. *Parasitology Research*, 103 (1), 29–43.
<https://doi.org/10.1007/s00436-008-1064-7>
- Hubálek, Z. & Halouzka, J. (1999) West Nile fever--a reemerging mosquito-borne viral disease in Europe. *Emerging Infectious Diseases*, 5 (5), 643–650.
<https://doi.org/10.3201/eid0505.990505>
- Ibáñez-Justicia, A., Smits, N., den Hartog, W., van de Vossenbergh, B., De Wolf, K., Deblauwe, I., Van Bortel, W., Jacobs, F., Vaux, A.G.C., Medlock, J.M. & Stroo, A. (2020) Detection of exotic mosquito species (Diptera: Culicidae) at international airports in Europe. *International Journal of Environmental Research and Public Health*, 17 (10), 3450.
<https://doi.org/10.3390/ijerph17103450>
- Jacobs, I., Feys, S., Nijs, D., Gielen, G., Verbelen, D. & Lambrechts, J. (2020) s.n. In: *Monitoring van ontsnipperings-maatregelen voor herpetofauna in Vlaanderen. Eindrapport. Rapport Natuurpunt Studie 9*. Mechelen, Belgium, pp. 82–89. Avail-

- able from: <https://archieff-algemeen.omgeving.vlaanderen.be/xmlui/handle/acd/262679> (accessed 26 August 2020)
- Lock, K., Dekoninck, W., Adriaens, T. & Goethals, P.L.M. (2012) Distribution and ecology of mosquito larvae (Diptera: Culicidae) in Flanders (Belgium). *Belgian Journal of Zoology*, 142 (2), 138–142.
- Lundström, J.O. (1999) Mosquito-borne viruses in western Europe: a review. *Journal of Vector Ecology*, 24 (1), 1–39.
- Medlock, J.M., Hansford, K.M., Versteirt, V., Cull, B., Kampen, H., Fontenille, D., Hendrickx, G., Zeller, H., Van Bortel, W. & Schaffner, F. (2015) An entomological review of invasive mosquitoes in Europe. *Bulletin of Entomological Research*, 25 (6), 1–27.
<https://doi.org/10.1017/S0007485315000103>
- Medlock, J.M. & Vaux, A.G.C. (2015) Impacts of the creation, expansion and management of English wetlands on mosquito presence and abundance—developing strategies for future disease mitigation. *Parasites & Vectors*, 8 (1), 142.
<https://doi.org/10.1186/s13071-015-0751-3>
- Papa, A., Danis, K., Baka, A., Bakas, A., Dougas, G., Lytras, T., Theocharopoulos, G., Chrysagis, D., Vassiliadou, E., Kamaria, F., Liona, A., Mellou, K., Saroglou, G. & Panagiotopoulos, T. (2010) Ongoing outbreak of West Nile virus infections in humans in Greece, July – August 2010. *Eurosurveillance*, 15 (34), 19644.
<https://doi.org/10.2807/ese.15.34.19644-en>
- Ponçon, N., Balenghien, T., Toty, C., Baptiste Ferre, J., Thomas, C., Dervieux, A., L’Ambert, G., Schaffner, F., Bardin, O. & Fontenille, D. (2007) Effects of local anthropogenic changes on potential malaria vector *Anopheles hyrcanus* and West Nile virus vector *Culex modestus*, Camargue, France. *Emerging Infectious Diseases*, 13 (12), 1810–1815.
<https://doi.org/10.3201/eid1312.070730>
- Radrova, J., Seblova, V. & Votýpka, J. (2013) Feeding behavior and spatial distribution of *Culex* mosquitoes (Diptera: Culicidae) in wetland areas of the Czech Republic. *Journal of Medical Entomology*, 50 (5), 1097–1104.
<https://doi.org/10.1603/ME13029>
- Randolph, S.E. & Rogers, D.J. (2010) The arrival, establishment and spread of exotic diseases: patterns and predictions. *Nature Reviews Microbiology*, 8 (5), 361–371.
<https://doi.org/10.1038/nrmicro2336>
- Reusken, C., De Vries, A., Den Hartog, W., Braks, M. & Scholte, E.J. (2010) A study of the circulation of West Nile virus in mosquitoes in a potential high-risk area for arbovirus circulation in the Netherlands, “De Oostvaardersplassen”. *European Mosquito Bulletin*, 28, 69–83.
- Ries, C. (2019) Three mosquito species new to Luxembourg: *Culex modestus*, *Uranotaenia unguiculata* and *Culiseta glaphyoptera*. Musée national d’histoire naturelle, Luxembourg. Available from: <https://mosquitoes.lu/three-mosquito-species-new-to-luxembourg-culex-modestus-uranotaenia-unguiculata-and-culiseta-glaphyoptera/> (accessed 26 August 2020)
- Rochlin, I., Faraji, A., Ninivaggi, D.V., Barker, C.M. & Kilpatrick, A.M. (2016) Anthropogenic impacts on mosquito populations in North America over the past century. *Nature Communications*, 7 (1), 13604.
<https://doi.org/10.1038/ncomms13604>
- Roiz, D., Ruiz, S., Soriguer, R. & Figuerola, J. (2015) Landscape effects on the presence, abundance and diversity of mosquitoes in Mediterranean wetlands. *PLoS One*, 10 (6), e0128112.
<https://doi.org/10.1371/journal.pone.0128112>
- Roiz, D., Wilson, A.L., Scott, T.W., Fonseca, D.M., Jourdain, F., Muller, P., Velayudhan, R. & Corbel, V. (2018) Integrated *Aedes* management for the control of *Aedes*-borne diseases. *PLOS Neglected Tropical Diseases*, 12 (12), e0006845.
<https://doi.org/10.1371/journal.pntd.0006845>
- Sohier, C. & Grootaert, P. (2015) *Muggenproblematiek van het Linkerscheldeoevergebied*. Royal Belgian Institute of Natural Sciences, Brussel, pp. 13–14.
- Thompson, R., Martin Del Campo, J. & Constenla, D. (2020) A review of the economic evidence of *Aedes*-borne arboviruses and *Aedes*-borne arboviral disease prevention and control strategies. *Expert Review of Vaccines*, 19 (2), 143–162.
<https://doi.org/10.1080/14760584.2020.1733419>
- Versteirt, V., Boyer, S., Damiens, D., De Clercq, E.M., Dekoninck, W., Ducheyne, E., Grootaert, P., Garros, C., Hance, T., Hendrickx, G., Coosemans, M. & Van Bortel, W. (2013) Nationwide inventory of mosquito biodiversity (Diptera: Culicidae) in Belgium, Europe. *Bulletin of Entomological Research*, 103 (2), 193–203.
<https://doi.org/10.1017/S0007485312000521>
- Versteirt, V., Nagy, Z.T., Roelants, P., Denis, L., Breman, F.C., Damiens, D., Dekoninck, W., Backeljau, T., Coosemans, M. & Van Bortel, W. (2015) Identification of Belgian mosquito species (Diptera: Culicidae) by DNA barcoding. *Molecular Ecology Resources*, 15 (2), 449–457.
<https://doi.org/10.1111/1755-0998.12318>
- Vlaskamp, D.R., Thijsen, S.F., Reimerink, J., Hilkens, P., Bouvy, W.H., Bantjes, S.E., Vlamincx, B.J., Zaaier, H., van den Kerkhof, H.H., Raven, S.F. & Reusken, C.B. (2020) First autochthonous human West Nile virus infections in the Netherlands, July to August 2020. *Eurosurveillance*, 25 (46), 2001904.
<https://doi.org/10.2807/1560-7917.ES.2020.25.46.2001904>
- Zeller, H., Lenglet, A. & Van Bortel, W. (2010) West Nile virus: the need to strengthen preparedness in Europe. *Eurosurveillance*, 15 (34), 19647.
<https://doi.org/10.2807/ese.15.34.19647-en>
- Zeller, H.G. & Schuffenecker, I. (2004) West Nile virus: An overview of its spread in Europe and the Mediterranean Basin

in contrast to its spread in the Americas. *European Journal of Clinical Microbiology and Infectious Diseases*, 23 (3), 147–156.

<https://doi.org/10.1007/s10096-003-1085-1>

Ziegler, U., Lühken, R., Keller, M., Cadar, D., van der Grinten, E., Michel, F., Albrecht, K., Eiden, M., Rinder, M., Lachmann, L., Höper, D., Vina-Rodriguez, A., Gaede, W., Pohl, A., Schmidt-Chanasit, J. & Groschup, M.H. (2019) West Nile virus epizootic in Germany, 2018. *Antiviral Research*, 162, 39–43.

<https://doi.org/10.1016/j.antiviral.2018.12.005>