OVERWINTERING OF CULEX MODESTUS AND OTHER MOSQUITO SPECIES IN A REEDBED ECOSYSTEM, INCLUDING ARBOVIRUS FINDINGS

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ABSTRACT. The overwintering strategy of the mosquito Culex modestus, an important West Nile virus (WNV) vector in Europe, was explored under field conditions in reedbed (Phragmites australis) ecosystems in early 2019. A total of 30 Cx. modestus females were found in a BG-Sentinel trap placed in a plastic greenhouse as well as in a reference BG-Sentinel trap placed under the open sky, both set up within the reedbeds and inspected every 2–3 days from February 27 to April 10, 2019. Moreover, 186 females of Cx. pipiens, 3 females of Anopheles hyrcanus, and 3 females of Culiseta annulata were trapped in the monitored time span. While all Cx. modestus females tested negative for the presence of WNV and other arboviruses circulating in Central Europe, we confirmed WNV lineage 2 and Ťahyna virus infection in several pools of the collected Cx. pipiens, demonstrating arbovirus overwintering. This pilot study highlights the need for large-scale monitoring activities covering different regions to identify the overwintering strategy of both mosquito-borne viruses and their vectors in Central Europe.

KEY WORDS Anopheles hyrcanus, Culex modestus, Culex pipiens, Culiseta annulata, overwintering, arboviruses

Culex modestus Ficalbi is a Eurasian mosquito species that is distributed from England to southern Siberia and common in southern and central countries of Europe (Becker et al. 2010). In Europe, its northern distribution range reaches northern Germany, where it is even found in metropolitan Hamburg (Krüger et al. 2014), Denmark (Bødker et al. 2014), parts of southern England (Golding et al. 2012), and southern Sweden, where this species was documented only recently (Lindström and Lilja 2018). Typical larval habitats of Cx. modestus include ponds and swamps with rich vegetation, marshes, flooded wetlands, ditches, and rice fields (Schaffner et al. 2001, Becker et al. 2010). In the Czech Republic, the species has been frequently reported from South Moravia and South Bohemia, where both larval and adult stages have been observed from early June to late September, typically in reedbeds surrounding fishponds (Vaňhara 1991, Votýpka et al. 2008, Šebesta et al. 2013), and often syntopically with Anopheles hyrcanus Pallas, An. maculipennis Meigen, Cx. pipiens Linnaeus, and Uranotaenia unguiculata Edwards (Šebesta et al. 2013).

Culex modestus is a very efficient vector of West Nile virus (WNV) (Balenghien et al. 2008, Cotar et al. 2016) and is also implicated in the transmission of Ťahyna and Lednice viruses (Danielová 1984, Hubálek and Halouzká 1996). The females of Cx. modestus feed on both birds (e.g., Anseriformes) and mammals (e.g., horses and rabbits) and may therefore act as bridge vectors for certain arboviruses (Balenghien et al. 2006, Rádrová et al. 2013, Brugman et al. 2017b). They are also reported to commonly bite humans outdoors, close to their larval habitat (Becker et al. 2010). According to Brugman et al. (2017a), Cx. modestus might be a significant human-biting species in certain areas of the United Kingdom.

In the Czech Republic, the origin of research on WNV (particularly its eco-epidemiology) dates back to 1997, when West Nile fever broke out for the 1st time, following catastrophic floods (Hubálek et al. 1999). Simultaneously, a so-called 3rd lineage of WNV (WNV-3 Rabensburg virus) was isolated from local Cx. pipiens (Hubálek et al. 1998). Further strains of Rabensburg virus were recovered in 1999 from Cx. pipiens commencing to overwinter (Hubálek et al. 2000), and again in 2006 from a specimen of mammal-feeding Aedes rossicus Dolbeskin, Goricaka, and Mitrofanova (Hubálek et al. 2014). Importantly, neuroinvasive WNV lineage 2 (WNV-2) strain was repeatedly isolated from Cx. modestus populations around local fishponds (Rudolf et al. 2014, 2015).
Very little is known about the overwintering habits of *Cx. modestus*. In southern France (the Rhône River delta, the Camargue), resting females were collected in December mainly from reed piles cut in autumn and covered by transparent plastic tents, but also directly from a reedbed, similarly covered (Mouchet et al. 1969). No data about overwintering of *Cx. modestus* (and other mosquito species occurring in reeds) are available for more temperate regions, including central and northern Europe. However, because of autumn activity of adults, absence of larvae during winter, and not cold-resistant eggs, it has been assumed that *Cx. modestus* hibernates as adult females in or around reedbed (*Phragmites australis* Cavanilles) ecosystems.

Detailed knowledge on the overwintering of mosquito vectors is crucial for understanding of WNV persistence in the temperate climatic zone. For this reason, we studied the overwintering of *Cx. modestus* under natural conditions in the Czech Republic.

The study was conducted at the “Nový rybník” fishpond (Fig. 1a), located in the Czech Republic, South Moravia, near the village of Sedlec (48°46’57”N, 16°40’13”E), which was already in the focus of previous studies (Šebesta et al. 2010; Hubálek et al. 2014; Rudolf et al. 2014, 2015). This location is well characterized by an established population of *Cx. modestus* occurring in a reedbed ecosystem and enzootic circulation of WNV during the mosquito season (Rudolf et al. 2014, 2015).

To examine the mode of *Cx. modestus* overwintering under natural conditions, we installed a plastic greenhouse (250 × 400 × 200 cm) in the fishpond reedbed in February 2019. The greenhouse was equipped with a BG-Sentinel trap (Biogents, Regensburg, Germany) connected to a gas bottle releasing CO₂ (200 ml/min) as an attractant (trap 1; Fig. 1b). In parallel, we installed 2 additional BG-Sentinel traps with CO₂, both placed freely, one in the middle of the reedbed (trap 2; Fig. 1c) and one in close proximity to the reeds (50 m) in neighboring woodland (trap 3; Fig. 1d). The traps were operated permanently from February 27 to April 10, 2019, and were inspected regularly 2–3 times/wk for the presence of adult mosquitoes. All collected mosquito specimens were stored at −60°C until their identification and further analyses. In addition, water and benthos were sampled on several occasions from the study site and inspected for the presence of mosquito larvae.

A total of 7 monospecific pools (1 pool of *An. hyrcanus*, 1 pool of *Culiseta annulata* (Schrank), 1
Table 1. Numbers of mosquito adult females collected from February 27 to April 1, 2019, in BG-Sentinel traps in reedbeds (traps 1 and 2) and adjacent woodland (trap 3) at the Nový rybník Pond, Sedlec, Czech Republic.

| Trap  | Culex modestus | Cx. pipiens | Anopheles hyrcanus | Culiseta annulata | Total |
|-------|----------------|-------------|-------------------|-------------------|-------|
| 1     | 7              | 10          | 3                 | 2                 | 22    |
| 2     | 23             | 176         | 0                 | 1                 | 200   |
| 3     | 0              | 12          | 0                 | 0                 | 12    |
| Total | 30             | 198         | 3                 | 3                 | 234   |

pool of Cx. modestus, and 4 pools of Cx. pipiens) consisting of 3–53 adult mosquito specimens were homogenized using tissue Lyser (Qiagen, Hilden, Germany) in 1.5–2.0 ml of cooled phosphate-buffered saline, pH 7.4, with 0.4% bovine serum albumin. Ribonucleic acid (RNA) was extracted from 150 µl of homogenates using the QIAamp viral RNA Mini Kit (Qiagen) according to the manufacturer’s instructions. All pools were tested for arboviruses recently demonstrated to circulate in the Czech Republic: Sindbis virus (SINV), Ťahýňa virus, and Usutu virus (USUV), and WNV. A continuous reverse transcriptase–polymerase chain reaction (RT-PCR) system employing the OneStep RT-PCR Kit (Qiagen) was applied on the RNA extracts. Oligonucleotide primers designed for SINV (Jöst et al. 2010), TAHV (Kuno et al. 1996), USUV (Bakonyi et al. 2004), WNV (Rudolf et al. 2014), and WNV-3 Rabensburg virus (Bakonyi et al. 2005) were used for the amplification. Specific PCR products were further processed by the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and characterized by Sanger sequencing on an ABI Prism 310 Genetic Analyzer (Applied Biosystems).

Since pertinent observations by Mouchet et al. (1969), Cx. modestus has been assumed to overwinter as adult females in reedbed ecosystems (e.g., in between dense reed stems, or inside the stems). To check for this mode of overwintering of Cx. modestus in the Czech Republic, we monitored reeds and their surroundings during winter and early spring. A total of 30 adult female Cx. modestus were caught in the installed traps (Table 1), with the 1st specimen collected on March 1, 2019. All of the Cx. modestus caught were trapped in the reedbed itself (traps 1 and 2), supporting the assumption of Mouchet et al. (1969). Nevertheless, alternative natural shelters, such as other dense vegetation, burrows of rodents in the ground, and tree and rock holes, might possibly be also used for overwintering. Interestingly, no Cx. modestus were caught in the reference trap 3 (situated in woodland), suggesting the habitat of Cx. modestus is narrowly restricted to reeds only.

Water samples were negative for aquatic stages of Cx. modestus throughout the study period, and overwintering has not been observed in the larval, pupal, or the egg stages. Despite this, egg longevity and survival under harsh conditions should be further studied in the laboratory.

Females of 3 additional mosquito species (An. hyrcanus, Cx. pipiens, and Cs. annulata) were found in the traps together with Cx. modestus (Table 1). Furthermore, many other arthropods belonging to several orders were found in aquatic samples or reared from the reed stems from the study site, e.g., spiders, psocopterans, hemipterans, hymenopterans, neuropterans, coleopterans, lepidopterans (moths), and dipterans (approximately 100 species). This confirms that reed stems are not only an important microhabitat for overwintering mosquitoes but also for a wide spectrum of other arthropod taxa, including phytophages, saprophages, predators, and parasitoids (Bogusch et al. 2016).

We demonstrated WNV-2 in 1 pool of Cx. pipiens mosquitoes with the highest sequence similarity (99.6%) to a previously found, locally circulating WNV strain (GenBank accession no. MF162729). Ťahýňa virus was found in 2 pools of Cx. pipiens with 99% sequence similarity to a TAHV strain originating from Italy (GenBank accession no. JN051146). The findings of both viruses in Cx. pipiens females that early in the season suggest their overwintering in the mosquitoes. We did not detect WNV (or any other pathogenic arbovirus) in overwintering Cx. modestus, so further research is needed to answer whether WNV is able to persist in Cx. modestus adults during winter in central or northern Europe, i.e., during periods of mosquito inactivity.

Exploring overwintering strategy of mosquito vectors is crucial for understanding of WNV persistence during an unfavorable season in Europe. Therefore, additional studies should include winter surveillance of larval habitats of Cx. modestus (as well as other mosquito vectors) in different parts of Europe where this species is frequently found, e.g., England, France, or Romania (Golding et al. 2012, L’Ambert et al. 2012, Cotar et al. 2016).

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