Feeding Behavior and Spatial Distribution of Culex Mosquitoes (Diptera: Culicidae) in Wetland Areas of the Czech Republic

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In Central Europe, serological surveys together with viral isolations indicate that mosquito-borne viruses such as Sindbis, West Nile, Usutu, Batai, and Tula are widespread (Hubálek et al. 2005). West Nile virus (WNV), a zoonotic mosquito-transmitted arbovirus whose enzootic cycle is maintained by birds and mosquitoes, was first detected in Bohemia and in several wetland areas in the Czech Republic. Culex pipiens (L.) and Culex modestus (F.) were the most frequently collected species. Although Cx. modestus did not distinguish between baits, Cx. pipiens was collected significantly more frequently in bird-baited traps. Based on mitochondrial DNA analysis of bloodmeals from engorged females collected by CO2-baited traps situated within reed beds, a diverse group of birds were the predominant hosts (93.7%), followed by mammals (4.2%) including humans, and amphibians (2.1%). Among birds, Anseriformes were fed upon most frequently by Cx. modestus, whereas Cx. pipiens fed most frequently on Passeriformes. To measure the infection risk and confirm the distribution of mosquito species in various biotopes, transects of CO2-baited CDC traps were operated from wetland reed beds into upland vegetated areas. Even though both Culex species occurred in all biotopes sampled and frequently dispersed hundreds of meters away from fishpond shore vegetation, the spatial distribution of Cx. modestus was significantly associated with reed beds at wetlands. The first detection of WNV (subtype RabV) in Cx. modestus in Bohemia and confirmation of WNV presence in Cx. pipiens in Moravia together with observed feeding behavior supports the presumed role of both Culex species in the avian-to-avian enzootic WNV cycle and in avian-to-mammal transmission in the Czech Republic.

KEY WORDS Culex, spatial distribution, WNV, Rabensburg virus, feeding preference
species have played an important role in European epidemics of WNV, it could be presumed that they are also involved in the WNV cycle in the Czech Republic. Herein, we report the spatial distribution, feeding behavior, and WNV infection of *Cx. pipiens* and *Cx. modestus* to identify possible virus foci in two selected wetlands areas of the Czech Republic.

**Materials and Methods**

**Collection Sites.** In our previous study focused on mosquito fauna (Votýpka et al. 2008), a 4-year (2004–2007) surveillance program was carried out at five separate wetland areas in the Czech Republic. Mosquitoes included in the current study were collected from July to September during the mosquito seasons of 2005 and 2006, at seven fish ponds situated in two wetland areas, to determine their feeding behavior, spatial distribution, and infection with WNV. Fishpond sites near Czech Budejovice (Černíš: 49° 0’ N, 14° 24’ E, 384 MSL [meters above sea level]: Zadní Topole: 49° 3’ N, 14° 22’ E, 386 MSL) and Písek (Blatěc: 49° 6’ N, 14° 18’ E, 393 MSL; Řezabinec: 49° 15’ N, 14° 5’ E, 380 MSL) were situated in southern Bohemia, whereas fish pond collection sites in southern Moravia were situated in the vicinity of Mikulov (Nestyt: 48° 46’ N, 16° 43’ E, 178 MSL; Nový: 48° 47’ N, 16° 40’ E, 192 MSL; Mušlov: 48° 48’ N, 16° 41’ E, 207 MSL). These two wetland and fishpond areas (southern Bohemia and southern Moravia) were popular recreation sites that host rich populations of migratory and resident birds, but are also very intensively farmed, because they are located in the fertile lowlands of the country. The immediate vicinity of the fishponds, where trap sites were situated, were usually not populated, and buildings, houses, or other permanent settlement were not present. The ponds were used primarily for medium-intensive fish farming; however, at the same time, the fishponds served as water-holding natural areas, and at least some of them are used for recreational purposes: two (Řezabinec and Nestyt) are protected as important bird areas. The areas surrounding the fishponds were used intensively for farming (fields, meadows, and vineyards), hunting (forests), fishing (ponds), and various recreational activities (including bird-watching etc.).

**Mosquito Collections.** Animal-baited traps were described previously (see Černý et al. 2011). In brief, two Centers for Disease Control and Prevention (CDC) traps (model 512 and 1012, John W. Hock Company, Gainesville, FL) without lights were placed on opposite faces of each animal-baited cage, consisting of a double wire cage (wire spacing 2 cm: inner cage: 50 by 40 by 30 cm; outer cage: 60 by 50 by 35 cm) to protect host animals against predators and with a Plexiglas roof to protect them against rain. Japanese quail (*Coturnix japonica*) were used as host birds, while scrub rabbits (*Oryctolagus cuniculus*) served as host mammals. Traps were set at around 1800 hours and collected the next morning at 0900 hours. Animals were placed in cages just before being transported to field sites and returned within an hour after removal of insects from the traps the next morning. Animals had continual access to food and water during insect trapping. The use of host animals was approved by the Ethical Committee of the Faculty of Sciences, Charles University (ČZU 945/05) and was carried out in accordance with the current laws of the Czech Republic.

All animal-baited traps were placed adjacent to fishponds overgrown with natural vegetation. At each locality, two pairs of animal-baited traps (thus four cages: two with rabbits and two with quails) were set for two nights. The host animals were interchanged (rabbit vs. quail) in traps during the two consecutive nights to avoid the influence of microclimate, micro-habitat, or both, on trap catch. A negligible number of *Culex* mosquitoes (up to five specimens per trap night), and significantly lower than in the case of animal-baited traps, were captured in 10 nonbaited cages placed =20 m away from animal-baited traps. These un-baited traps served as negative controls. To confirm that mosquitoes actually fed on the animal baits, the bloodmeals from 60 blood-engorged females of *Cx. pipiens* and *Cx. modestus* captured in mammalian and bird-baited traps (15 specimens of each combination) were analyzed. The DNA analysis of this blood corresponded with the animal species used for bait in all 60 samples.

The spatial distribution of host-seeking mosquito females was studied by using CDC traps baited with dry ice (CO2). These traps were hung 1 meter above ground level on transect lines radiating outward from the central ponds to determine the risk of contact with *Culex* mosquitoes in various biotopes surrounding the fishponds. In total, 36 trap nights positioned along 13 transect lines were placed in both wetland areas: five in southern Bohemia (fishponds Blatěc and Řezabinec) and eight in southern Moravia (fishponds Nestyt, Nový, and Mušlov). The number of CDC traps in transects varied from three to six depending on the heterogeneity of the site. All transects were perpendicular to fishpond shorelines, and each trap was positioned within a different biotope at =30-m intervals. For statistical measurement, the traps were divided into four categories according to biotope and distance from water shorelines: 1) reed beds and other vegetated areas surrounding the fishponds (14 trap nights), 2) transitional areas (ecotones) between reeds and surrounding biotopes (13 trap nights), 3) neighboring biotopes such as meadow and field (13 trap nights), and 4) distant biotopes such as forest (16 trap nights).

**Mosquito Processing and Species Identification.** Mosquitoes were killed with dry ice and stored at −70°C. In the laboratory, mosquitoes were enumerated by species (see Votýpka et al. 2008), sex, and blood feeding status under a stereomicroscope on a chill table. Bloodmeals of engorged females were expressed into filter paper (Whatman no. 3) and stored at −20°C until DNA extraction. Unfed females were grouped into pools (from one to 50 specimens separated by species, locality, and date), stored at −70°C,
and later tested for WNV by reverse transcription-polymerase chain reaction (RT-PCR).

**Bloodmeal Identification.** Because the majority of blood-fed females were captured by CO₂-baited CDC traps in reed beds surrounding fishponds, bloodmeal identification was based exclusively on mosquitoes trapped in this habitat (category 1, see above). To avoid the influence of Japanese quail and rabbit blood on bloodmeal identification, the animal-baited traps were used in different localities, on different days than trapping, or both, to provide blood-fed females for bloodmeal analyses.

Total DNA of blood-engorged females, partially analyzed in our previous study (Votýpka et al. 2008), was extracted according to manufacturer protocols (High pure PCR template preparation kit, Roche, Mannheim, Germany). Bloodmeals were identified by direct sequencing of an ~350 bp segment of the cytochrome b (cyt b) gene on an automated DNA sequencer (310 Genetic Analyzer; ABI Prism, Foster City, CA) using the BigDye 3.1 kit (Applied Biosystems, Foster City, CA). Universal vertebrate primers cyt bb1 (5′-CCG TCM AAC ATY TCA DTA TGA AA-3′) and cyt bb2 (5′-GCH CCT CAG AAT GAY ATT TGK CCT CA-3′) were used with the following cycling profile: 94°C for 5 min, 35× (94°C for 1 min, 55°C for 1 min, 72°C for 1 min), and 72°C for 7 min. Sequence analyses were performed using DNASTAR software (DNASTAR, Inc., Madison, WI) and compared with sequences deposited in the GenBank database using standard nucleotide BLAST searches. The method was not able to reliably identify samples with mixed blood sources. To determine the duration of DNA persistence after blood feeding, colonized *Culex quinquefasciatus* Say were allowed to feed on anesthetized mice. Time course analysis on amplification of the cyt b gene showed that the host DNA could be detected up to three days after blood feeding under laboratory conditions (20–22°C, 80% relative humidity).

**WNV Detection.** An RNA QIAamp viral mini kit (Qiagen, Hilden, Germany) was used for RNA extraction. Reverse transcription to cDNA was performed by SuperScriptIII Reverse Transcriptase (Invitrogen, MD) with random hexamers (Promega, WI) according to the manufacturer’s protocol. Two PCR amplifications were performed simultaneously using primers specific for the WNV env region: WN233 and WN640c (Lanciotti et al. 2000) and RabV primers, RAB233 (5′-TCGATGGCCTTGGGATTC-3′) and RAB640c (5′-CTCGCGGCAAAGCTGGC-3′), amplifying a segment 408 bp long with the following cycling profile: 45°C for 60 min, 94°C for 3 min, 45 × (94°C for 30 s, 60°C for 1 min, 68°C for 3 min), and 72°C for 7 min. Positive samples were confirmed by direct sequencing as described above.

**Data Analysis.** Collections of mosquitoes from host-baited traps were normalized using a Log₁₀ transformation and analyzed using generalized linear models (GLM; STATISTICA 6.0, StatSoft, Inc., Tulsa, OK), with respect to collection sites and seasons as main effects. Multivariate analyses of *mosquito* spatial distributions were performed with the software package CANOCO for Windows v. 4.5 (Braak and Šmilauer 2002, Petrušek et al. 2008). Original counts (number of individuals) were log transformed, and standardized by sample norm was used to focus the analyses on the differences in the relative proportion of individual taxa (*Cx. pipiens* and *Cx. modestus* species and *Aedes, Culiseta, Mansonia*, and *Anopheles* genera). To summarize and visualize occurrence patterns of mosquito taxa and the relationship between species composition and the spatial gradient (distance from the fishpond shorelines), principal component analysis (PCA) was used. Analysis of frequencies for the bloodmeal source of engorged females from CO₂ traps was done using Pearson’s χ² test (STATISTICA).

### Results

**Animal-Baited Traps.** One of the study’s aims was to determine the host-seeking behavior of mosquitoes in wetland areas in the Czech Republic. In 2005 and 2006, a total of 29,923 mosquitoes of 14 species belonging to five genera were collected during 152 trap nights using animal-baited traps. Overall species abundance of mosquitoes in the same areas was partially analyzed in our previous study (Votýpka et al. 2008), which demonstrated no significant differences between years (2005 vs. 2006). In the current study, no significant differences between years (2005 vs 2006) were found in species abundance for animal-baited traps (GLM; *F*₁,₃₁ = 0.80; *P* > 0.05).

In total, 15,099 and 14,824 mosquitoes were caught by Japanese quail-baited and rabbit-baited traps, respectively (Table 1). The most frequently collected species were *Cx. modestus* and *Cx. pipiens*, followed by *Aedes cinereus* Meigen and *Aedes vexans* Meigen. Overall, there were no significant differences between the number of mosquitoes captured by bird versus mammal-baited traps (numbers per trap night with traps replicated over time and space [main effects: seasons and sites]; GLM: *F*₁,₃₁ = 0.8; *P* > 0.05). Although *Cx. modestus* were not significantly attracted to quail versus rabbit (GLM; *F*₁,₃₁ = 0.1; *P* > 0.05), *Cx. pipiens* was collected significantly more frequently at quail-baited than at rabbit-baited traps (GLM; *F*₁,₃₁ = 15.4; *P* < 0.001). Statistical analyses of the two most abundant *Aedes* species did not reveal significant differences in collection at the two host-baited traps (*Ae. vexans*: GLM; *F*₁,₃₁ = 0.29; *P* > 0.05; *Ae. cinereus*: GLM; *F*₁,₃₁ = 1.98; *P* > 0.05).

**Bloodmeal Identification.** Out of 93,865 female mosquitoes captured by CO₂-baited CDC traps placed in reed beds surrounding pond shores during both the present and previous (Votýpka et al. 2008) studies, 159 females (0.17%) contained some blood in their gut: 97 females (0.17%) contained some blood in their gut: 97 *Cx. pipiens*, 50 *Cx. modestus*, 7 *Ae. vexans*, 3 *Gq. richiardii*, and 2 *Anopheles maculipennis*. Only data for the two most abundant mosquito species, *Cx. pipiens* and *Cx. modestus*, were analyzed. The bloodmeal source was determined for 95 *Culex* females (a success rate of 65%), and 35 different host species were identified. The majority (93.7%) of bloodmeals came from birds.
(89 blood samples belonging to 30 bird species). Four bloodmeals originated from three mammalian species, and two bloodmeals were from amphibians (Table 2).

The success of bloodmeal identification was independent on mosquito species ($\chi^2 = 2.8; \text{df} = 1; P > 0.05$). Both Culex species fed mainly on Anseriformes and Passeriformes. Whereas Cx. modestus fed nearly equally on both bird orders. Cx. pipiens fed most frequently on Passeriformes ($\chi^2 = 12.90; \text{df} = 1; P < 0.001$). No significant differences between seasons (2005 vs 2006; $\chi^2 = 2.06; \text{df} = 2; P > 0.05$) or areas (southern Bohemia vs southern Moravia; $\chi^2 = 2.32; \text{df} = 2; P > 0.05$) were observed.

**Spatial Distribution.** During 2006 and 2007, transects of CO2 traps were used to determine mosquito spatial distribution and their occurrence in various biotopes according to distance from ponds. We found host-seeking females in all studied biotopes, including upland vegetated areas occasionally far from pond shorelines, the presumed breeding sites of Cx. modestus and Cx. pipiens. In total, 12,110 mosquitoes of 13 species belonging to five genera were caught using 13 transect lines placed in five localities (56 trap nights). For statistical measurement, traps were divided into four categories according to the distance from shorelines. Exploratory analysis (STATISTICA) showed that the proportion of both Culex species (data not shown) and the number of mosquito females captured per trap night depended on the distance. Despite the fact that Cx. pipiens generally dominated in all four biotopes, Cx. modestus was more abundant in reed beds at wetlands and neighboring biotopes (Fig. 1). However, both Culex species were present even in traps at more distant biotopes, situated as far as 200 m away from the shore.

Similarly, PCA analysis (CANOCO) revealed a strong correlation ($P < 0.05$) between the occurrence of mosquitoes and the distance from pond shorelines. According to this analysis, distance explained 16% of the species composition variability (with the rest explained by locality, season etc.). Whereas the occurrence of Cx. pipiens was slightly positively correlated with distance, Cx. modestus demonstrated a strong negative correlation with the distance from shorelines (Fig. 2). Aedes species did not correlate with distance; this corresponds well with the fact that reed beds are not a larval habitat for these species.

**Virus Detection.** In total, 8,726 mosquito females belonging to three species were divided into 188 pools...
and tested for WNV: 64 Cx. pipiens pools (35 from southern Bohemia and 29 from southern Moravia), 118 Cx. modestus pools (93 and 25), and 6 Cq. richiardii pools (0 and 6). Virus was detected in 11 pools: seven WNV-positive pools of Cx. pipiens originated from three collection sites (Nesyt, Muslov, Nový) and two catching seasons (2006 and 2007) in southern Moravia, whereas in southern Bohemia four WNV-positive pools of Cx. modestus originated just from one collection site (Řezábinec) in 2006 and represent the first detection of WNV in Bohemia. PCR products observed on agar gels were confirmed by sequencing. In all cases, the virus was identified as Rabensburg virus (lineage three of WNV). The nucleotide substitutions of 11 newly obtained WNV Rabensburg sequences are summarized in Table 3.

Discussion

The current study describes the occurrence, spatial distribution, and feeding behavior of two Culex mos-
feeding behavior of central European hosts. Fyodorova et al. (2006) described collected engorged females fed only on birds, proboscis (53.4%) or Japanese quail (46.6%), but field-estus did not exhibit a preference for either caged Fyodorova et al. 2006). In the current study, regions are opportunistic and feed on birds as well as populations, although populations in other European regions are opportunistic and feed on birds as well as vertebrates like frogs, lizards, and snakes (Apperson et al. 2004, Lura et al. 2012). Our previous study demonstrated that Cx. modestus has spread throughout the Czech Republic in recent years (Votyka et al. 2004, Molaei et al. 2006), which was confirmed by using animal-baited traps and sequencing of engorged blood in the current study. Our previous study demonstrated that Cx. modestus has spread throughout the Czech Republic in recent years (Votyka et al. 2008). Little is known about the feeding behavior of central European Cx. modestus populations, although populations in other European regions are opportunistic and feed on birds as well as mammals, including humans (Bulenghien et al. 2006, Fyodorova et al. 2006). In the current study, Cx. modestus did not exhibit a preference for either caged rabbits (53.4%) or Japanese quail (46.6%), but field-collected engorged females fed only on birds, probably because of the low number of suitable mammalian hosts. Fyodorova et al. (2006) described Cx. modestus as being ornithophagic as did Minář (1969) who studied the feeding behavior of Cx. modestus in southern Moravia using sentinel hosts. As a consequence of the recent geographical spread of Cx. modestus within Bohemia (Votyka et al. 2008) and its willingness to feed on both avian and mammalian hosts, the species appears to be a potential bridge vector of WNV in the Czech Republic. This is supported by this study’s detection of WNV in four Cx. modestus pools obtained from southern Bohemia.

The third mosquito species, Cq. richiardii, which has been considered to be a vector during several European WNV outbreaks (Hubálek and Halouzka 1999, Savage et al. 1999), is rare in the Czech Republic (Votyka et al. 2008). Our data confirmed this conclusion, with only 19 specimens equally entering mammalian and bird-baited traps. The relatively low density of Cq. richiardii throughout the Czech Republic prevents it from being an important vector in WNV transmission in central Europe, as stated by Balenghien et al. (2006).

Interspecific differences in mosquito bloodmeal composition have an important effect on the potential transmission risk of WNV to birds and mammals including humans. The identification of bloodmeals from females engorged on wild animals (30 bird, 3 mammalian, and 2 amphibian species) indicated a broad range of avian blood sources used by Culex mosquitoes, although significant differences in the proportion of the bird orders Anseriformes and Passeriformes were detected in Cx. pipiens and Cx. modestus bloodmeals. Whereas Cx. pipiens fed more frequently on Passeriformes (e.g., Turdus and Sturnus), Cx. modestus focused on Anseriformes (e.g., Anas and Anser). Such disparity could be explained either by a difference in host-seeking behavior or by different mosquito and avian host occurrences in various microhabitats. Despite the fact that all analyzed blood-fed females were captured in reed beds, this does not mean that mosquitoes fed on their hosts in this particular biotope. Cx. modestus is generally more restricted to reed beds at wetlands where Anseriformes frequently occur, whereas Passeriformes frequented distant biotopes where a higher proportion of Cx. pipiens were collected. A similar pattern has been found in many other studies (Ngo and Kramer 2003, Apperson et al. 2004, Lura et al. 2012, Roiz et al. 2012) describing Passeriformes as the most frequent host of Cx. pipiens. As we do not have data on bird abundance at the sites studied here, we are unable to measure the host genus or species preference as has been done, for example, by Lura et al. (2012). Even though blood-

### Table 3. Nucleotide substitutions of WNV—Rabensburg sequences of WNV env region, which were obtained from 11 mosquito pools collected in 2006 and 2007 in southern Bohemia (Režabinec—Re) and southern Moravia (Nesy—Nes, Mušlov—Mu, and Nový—Nr)

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Positions refer to the complete genome sequence of WNV Rabensburg strain 97–103 (GenBank AY652464) and two additional strains, 99–222 (GQ421358) and 06–222 (GQ421359). All three reference strains were isolated from mosquitoes captured in southern Moravia.
meal identifications confirmed our results from animal-baited traps, mammalian blood was found in only a small proportion of engorged Cx. pipiens females captured by CO₂-baited traps. No mammalian blood was detected in Cx. modestus, possibly because of the unavailability of wild mammalian hosts in reed beds within wetlands.

The main aim of the transect sampling was to survey the abundance of mosquitoes in different biotopes at increasing distances from shorelines, and to delineate the probability of WNV transmission in the vicinity of fishponds. PCA analysis (CANOCO) showed that Cx. pipiens and Cx. modestus species were not only associated with reed beds, the presumed breeding sites of Cx. modestus, but also were abundant in upland biotopes hundreds of meters away from fish ponds and reed beds (e.g., in meadows, forests, fields, and vineyards). However, considerable differences were observed in abundance patterns between Culex species, because Cx. modestus significantly preferred reeds. Differences detected in the spatial distribution of the two Culex species could be explained by various larval habitats. Whereas Cx. modestus preferred ponds as oviposition sites (Mouchet et al. 1970), the larval habitats for Cx. pipiens could be scattered in different sites and microhabitats. Even though we did not search for potential mosquito larva habitats, in water reservoir plastic traps (∼20 bowls 45 by 45 by 20 cm) positioned randomly in various biotopes, only Cx. pipiens larvae were detected. Our finding of Cx. modestus in reed beds is in accordance with the study of Mouchet et al. (1970), who showed a high density of host-seeking Cx. modestus females in reeds, marshes, and riverine forests in Camargue, France. Even though a similar observation was made by Miñàr (1969) who surveyed the frequency of Cx. modestus feeding on humans at different distances, all Cx. modestus in his study were observed within 10 m of areas with reed beds. In our experiment, we collected host-seeking females, of which both species were up to 200 m upland from reed beds. This divergence can most likely be explained by different sampling methods (5-min subject exposures vs. overnight exposures of CO₂-baited CDC traps in our study). The abundance of host-seeking females of another Culex species, Culex tarsalis, was also shown to be higher at upland vegetation ecotones, and whose presence increased with distance from the breeding site (Lothrop and Reisen 2001). However, it is clear that Culex mosquitoes can be found in more distant biotopes. During mark–release–recapture studies performed in California, Culex stigmatosoma was recaptured 4.3 km and Cx. tarsalis 6.1 km from their release point, but the majority of marked host-seeking females were recaptured within 1 km (Reisen et al. 1991, 1992; Reisen and Lothrop 1995). The occurrence of Cx. pipiens and Cx. modestus species in distant biotopes allows us to speculate about a comparatively high risk of WNV infection for hunters, farmers, and other people residing in biotopes surrounding ponds, as well as for farm animals, mainly horses.

WNV was detected in 11 pools of Culex species, which supports previous reports that Cx. pipiens and Cx. modestus are the principal vectors of WNV in central Europe. Based on sequencing, all of our findings are Rabensburg virus (RabV; subtype of WNV; lineage 3), previously isolated in southern Moravia from Cx. pipiens in 1997 and 1999 and from Aedes rossicus Dolbeskin & Goricajka in 2006 (Hubálek et al. 1998, 2010; Hubálek 2000). Our results suggest the occurrence of WNV in additional localities, as the virus was detected for the first time in mosquitoes (Cx. modestus) captured in Bohemia; however, it could be speculated that owing to virus detection only in one collection site during one season, the spread of the virus in Bohemia is far more limited than in Moravia. We believe that our findings are important for surveillance programs focusing on pathogenic agents transmitted by mosquitoes. Future studies should be focused on WNV detection in mosquitoes and wild and domestic birds in more localities in Bohemia to identify possible transmission foci as well as the vector competence of Cx. modestus for WNV should be tested.

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