

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

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ABSTRACT Mosquito feeding behavior determines the degree of vector–host contact and may have a serious impact on the risk of pathogen transmission, including that of the West Nile virus (WNV). To measure the role of *Culex* mosquitoes as WNV vectors, host-seeking females were collected using animal-baited traps containing live birds (quail) or mammals (rabbits) and CO₂-baited Center for Disease Control and Prevention traps placed 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

In Central Europe, serological surveys together with viral isolations indicate that mosquito-borne viruses such as Sindbis, West Nile, Usutu, Batai, and Ťahýňa are widespread (Hubálek et al. 2005). West Nile virus (WNV) is a zoonotic mosquito-transmitted arbovirus whose enzootic cycle is maintained by birds and mosquitoes (Hubálek and Halouzka 1999). In recent decades, human outbreaks of WNV have been reported in many European countries (Savage et al. 1999, Platonov et al. 2001, Mailles et al. 2003, Hellenic Centre for Disease Control and Prevention [HCDCP] 2011), and since 1997, human cases as well as WNV-infected mosquitoes, have been reported from southern Moravia, the Czech Republic (Hubálek and Halouzka 1999; Hubálek 2000; Hubálek et al. 2005, 2010), but, before the current study, not from Bohemia. The virus strain isolated from *Culex pipiens* (L.) collected in southern Moravia in 1997 and named Rabensburg (RabV) was

considered to represent a novel lineage (lineage 3) of WNV (Bakonyi et al. 2005).

Among the mosquito species collected during European WNV outbreaks, *Cx. pipiens* (L.), *Culex modestus* (F.), and *Coquillettidia richiardii* (F.) have been considered the main vectors, based on their abundance, feeding behavior, virus infection, and vector competence (Hubálek and Halouzka 1999; Balenghien et al. 2006, 2007). These mosquito species are involved in enzootic transmission among birds; however, their role as bridge vectors to mammals including equines and humans is still unclear (Hubálek and Halouzka 1999). Monitoring vector abundance is the first step toward understanding virus ecology and transmission cycles. The expanding distribution of *Cx. modestus*, an important WNV vector, as well as the spread of other mosquito species in the Czech Republic, has been recently reported (Votýpka et al. 2008, Sebesta et al. 2012).

Vector behavior and vectorial capacity are both critical aspects of pathogen transmission. Feeding preferences and spatial distribution of host-seeking mosquitoes influence the spectrum of host contacts, and therefore their role as vectors of animal and human pathogens. Because both above-mentioned *Culex*

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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 České Budějovice (Černíř: 49° 0' N, 14° 24' E, 384 MSL [meters above sea level]; Zadní Topole: 49° 3' N, 14° 22' E, 386 MSL) and Písek (Blatec: 49° 6' N, 14° 18' E, 393 MSL; Řežabinec: 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 (Řežabinec 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, microhabitat, 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, 56 trap nights positioned along 13 transect lines were placed in both wetland areas: five in southern Bohemia (fishponds Blatec and Řežabinec) 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'-CCA TCM AAC ATY TCA DCA TGA TGA AA-3') and *cyt bb2* (5'-GCH CCT CAG AAT GAY ATT TKG 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 (Lancioti et al. 2000) and RabV primers, RAB233 (5'-TCGTATTGGCCCTATTGGCATTCTT-3') and RAB640c (5'-CTGCGGCAAGCACTGGACATTCATA-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 dis-

tributions 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 χ^2 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 *Cx. pipiens*, 50 *Cx. modestus*, 7 *Ae. vexans*, 3 *Cq. 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

Table 1. Mosquitoes collected by animal-baited traps in 2005 and 2006: total number of collected mosquito females (Total no.), mean number of collected mosquitoes per one trap night (mean) \pm SE, and proportion of the total catch (%)

Mosquito species	Japanese quail (76 trap nights)			Rabbit (76 trap nights)		
	Total no.	Mean \pm SE	%	Total no.	Mean \pm SE	%
<i>Culex modestus</i>	10,583	278.5 \pm 91.7	70.1	12,134	319.3 \pm 111.6	81.9
<i>Culex pipiens</i>	4,118	108.4 \pm 20.6	27.3	1,783	46.9 \pm 13.1	12.0
<i>Aedes vexans</i>	154	4.1 \pm 1.2	1.0	360	9.5 \pm 3.8	2.4
<i>Aedes cinereus</i>	167	4.4 \pm 1.7	1.1	459	12.1 \pm 3.5	3.1
<i>Aedes cantans</i>	3			5		
<i>Aedes communis</i>	2			16		
<i>Aedes sticticus</i>	42			26		
<i>Aedes leucomelas</i>	0			1		
<i>Anopheles maculipennis</i>	2			3		
<i>Anopheles claviger</i>	0			3		
<i>Anopheles hyrcanus</i>	9			12		
<i>Coquilletidia richiardii</i>	10			9		
<i>Culiseta annulata</i>	9			12		
<i>Culiseta morsitans</i>	0			1		

(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 ($\chi^2 = 2.8$; 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 ($\chi^2 = 12.90$; df = 1; $P < 0.001$). No significant differences between seasons (2005 vs 2006; $\chi^2 = 2.06$; df = 2; $P > 0.05$) or areas (southern Bohemia vs southern Moravia; $\chi^2 = 2.32$; 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

Table 2. Bloodmeal source of *Culex* females collected in southern Bohemia and Moravia during 2005 and 2006 mosquito season

Host (scientific names)	Host (common names)	<i>Cx.</i> <i>pipiens</i>	<i>Cx.</i> <i>modestus</i>
<i>Anas platyrhynchos</i>	Mallard	2	8
<i>Anas strepera</i>	Gadwall		1
<i>Anas</i> sp.	Duck <i>Anas</i> sp.	1	
<i>Anser anser</i>	Greylag Goose	2	10
<i>Anser albifrons</i>	Greater White-fronted Goose	1	
<i>Anser</i> sp.	Goose <i>Anser</i> sp.	1	
<i>Aythya</i> sp.	Ducks <i>Aythya</i> sp.	1	
Total (Anseriformes)		8	19
<i>Acrocephalus scirpaceus</i>	Eurasian Reed-warbler	1	
<i>Delichon urbica</i>	Northern House-martin	5	
<i>Emberiza citrinella</i>	Yellowhammer	2	3
<i>Erethacus rubecula</i>	European Robin		1
<i>Fringilla coelebs</i>	Eurasian Chaffinch	1	3
<i>Hirundo rustica</i>	Barn Swallow	3	1
<i>Motacilla alba</i>	White Wagtail	1	
<i>Muscicapa striata</i>	Spotted Flycatcher		1
<i>Parus caeruleus</i>	Blue Tit	2	1
<i>Parus major</i>	Great Tit		1
<i>Passer domesticus</i>	House Sparrow		1
<i>Pica pica</i>	Black-billed Magpie	1	
<i>Sturnus vulgaris</i>	Common Starling	6	1
<i>Sylvia atricapilla</i>	Blackcap	1	
<i>Sylvia communis</i>	Common Whitethroat	3	
<i>Turdus merula</i>	Eurasian Blackbird	6	1
<i>Turdus philomelos</i>	Song Thrush	4	
Total (Passeriformes)		36	14
<i>Coturnix coturnix</i>	Common Quail	5	
<i>Nycticorax nycticorax</i>	Black-crowned Night-heron		1
<i>Circus aeruginosus</i>	Western Marsh-harrier	1	2
<i>Rallus aquaticus</i>	Water Rail	1	
<i>Ardea cinerea</i>	Grey Heron	1	
<i>Phasianus colchicus</i>	Common Pheasant	1	
<i>Homo sapiens</i>	Human	2	
<i>Felis catus</i>	Cat	1	
<i>Nyctalus noctula</i>	Common Noctule	1	
<i>Rana</i> sp.	Common frog <i>Rana</i> sp.	1	
<i>Hyla arborea</i>	European tree frog	1	
Total (identified)		59	36

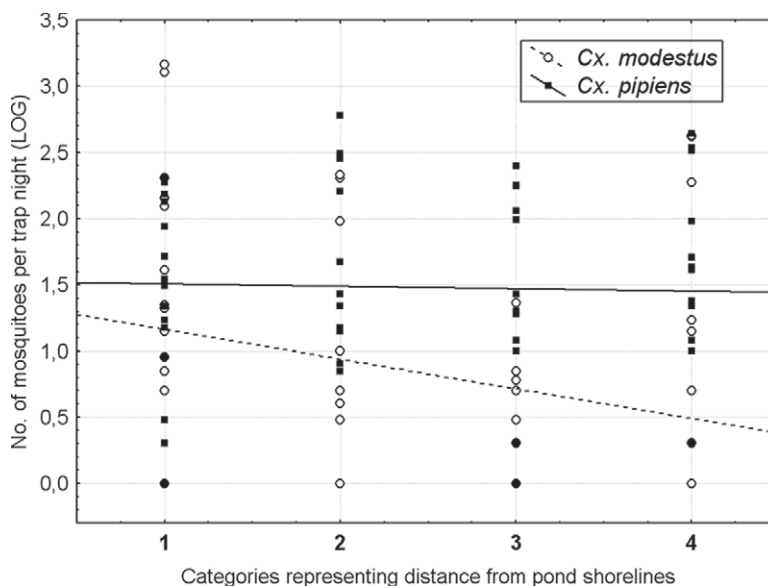


Fig. 1. Scatterplot of numbers of *Cx. pipiens* and *Cx. modestus* females captured per trap night by using transect lines. The traps were divided into four categories according to biotope and distance from water shorelines: 1) reeds (up to 50 m), 2) boundary of reeds with surrounding biotopes, 3) neighboring biotopes (up to 100 m), 4) distant biotopes (over 100 m).

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, Mušlov, 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 (Řežabinec) 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-

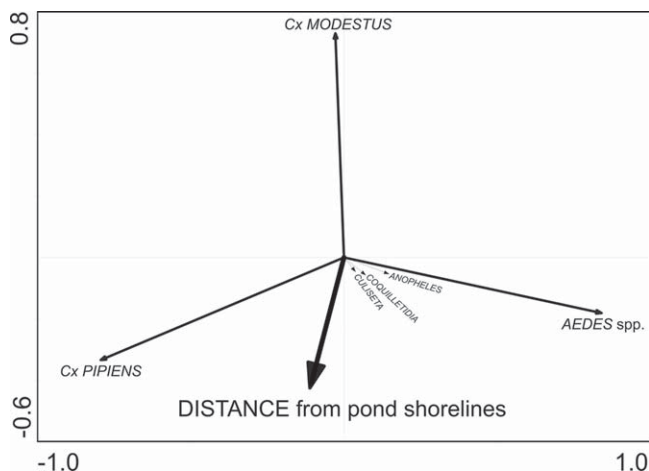


Fig. 2. Relationships between abundance of mosquito species and distance from fishpond shorelines. Results were obtained by partial redundancy analysis of the spatial distribution of mosquito species along the distance from breeding sites using principal component analysis (PCA; CANOCO). Data from 2005 and 2006 were pooled. The length and position of the arrows indicate the strength of the relationship. Arrows indicate tendencies in the occurrence of the appropriate taxa. The relationship of each variable is equal to the angle between the arrows; a small angle (hence concordant direction) indicates a positive correlation (e.g., *Cx. pipiens*), the opposite direction indicates a negative correlation (e.g., *Cx. modestus*).

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 (Řežabinec—Re) and southern Moravia (Nesyt—Nes, Mušlov—Mu, and Nový—Nr)

Position (strain 97–103)	260	306	318	357	369	404	408	419	453	582	609
97–103 1997 <i>Cx. pipiens</i>	A	A	A	A	C	A	A	C	T	A	C
99–222 1999 <i>Cx. pipiens</i>	T
06–222 2006 <i>Ae. rossicus</i>	G	.
Re-1–2006 <i>Cx. modestus</i>	G	.	G	G	.
Re-2–2006 <i>Cx. modestus</i>	G	.	G	G	.
Re-3–2006 <i>Cx. modestus</i>	G	.	G	G	.
Re-4–2006 <i>Cx. modestus</i>	G	.	G	G	.
Nr-1–2006 <i>Cx. pipiens</i>	G	.	G	G	A	G	.
Nr-2–2007 <i>Cx. pipiens</i>	G	.	G	G	.
Nr-8–2007 <i>Cx. pipiens</i>
Nes-1–2007 <i>Cx. pipiens</i>	G	.	G	.	.	.	G	T	.	G	.
Mu-1–2006 <i>Cx. pipiens</i>	G	T	G	.	.	G	.	.	.	G	.
Mu-2–2006 <i>Cx. pipiens</i>	G	.	G	.	T	G	.
Mu-3–2006 <i>Cx. pipiens</i>	G	.	G	G	.

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.

quito species associated with WNV transmission (*Cx. pipiens* and *Cx. modestus*) at several fishponds in the Czech Republic. Based on animal-baited traps, *Cx. pipiens* showed a greater degree of ornithophagy than *Cx. modestus*. Similar feeding behavior has repeatedly been described in numerous publications, and it appears that *Cx. pipiens* avian host preferences are rather wide (Lura et al. 2012, Munoz et al. 2012, Osório et al. 2012) and change with collection site and host availability (Apperson et al. 2004, Munoz et al. 2012). Our findings of frog blood agreed with other studies that described occasional feeding on cold-blooded vertebrates like frogs, lizards, and snakes (Apperson et al. 2002, Medlock et al. 2005, Munoz et al. 2012). *Cx. pipiens* is generally considered predominantly ornithophilic, but is willing to feed on mammals (Apperson 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 (Votýpka 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 (Balenghien 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 ornithophilic 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 (Votýpka 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 (Votýpka 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-

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 Camarque, France. Even though a similar observation was made by Minář (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 in the Minář study 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 & Gorickaja 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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