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Mosquitoes and biting midges as vectors in the Czech Republic

Komáři a tiplíci jako přenašeči v České republice

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I declare, that most of the work presented in this thesis was accomplished by myself. This thesis was written by myself, all the literary sources were properly cited, and it has not been used as a final work towards any other university degree.

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

Praha, 3.8.2015 Jana Rádrová

I declare, that most of the work presented in this thesis are results of Jana Rádrová. Jana has substantially contributed to the field and experimentally work and as well as the writing of the manuscripts.

Prohlašuji, že většina výsledků prezentovaných v této práci byla dosažena Janou Rádrovou. Jana se významně podílela na terénní a experimentální práci i na sepsání prezentovaných publikací.

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Contents

Abstract

Insects are the largest and most diverse class of animals. Many species have a significant impact on people and their activities, whether positive or negative. Since the late 19th century, it has been known that bloodsucking insects can serve as vectors of pathogens, causative agents of many infectious diseases. As the climate changes, the distribution and abundance of arthropods including bloodsucking insects can be affected. The emergence of new vector-borne diseases in Europe is likely to be among the most important effects of global changes. In recent years, several vector-borne diseases affecting humans and domestic animals have (re)emerged and spread in Europe, with major health, ecological and socio-economical consequences. For example, the mosquito-borne West Nile virus affects both human and animal health. Also, two new zoonosis have emerged, caused by Bluetongue and Schmallenberg viruses and transmitted by biting midges, affecting mainly small domestic ruminants.

In the frame of broader projects, two epidemiological and entomological surveys focused on mosquitoes and biting midges as possible vectors of West Nile virus, Bluetongue or Schmallenberg virus were carried out in the Czech Republic. New mosquito and biting midge species were recorded for the Czech Republic, and the presence of West Nile virus was found in mosquitoes as well as a new trypanosome species, *Trypanosoma culicavium*. Aside from the faunistic studies and screening for pathogen presence in insects, we also studied the feeding preferences of mosquitoes, which strongly correlate with parasite transmission as they influence the spectrum of the host contacts.

Abstrakt

Hmyz je jednou z nejrozmanitějších a nejrozšířenějších skupin živočichů, v rámci které nalézáme mnoho druhů ovlivňujících lidi, ať pozitivně či negativně. Již od konce 19. století je známo, že krevsající hmyz může hrát roli v přenosu patogenů působících různá onemocnění. Spolu se změnami klimatu dochází také k šíření členovců, včetně krevsajícího hmyzu na nová území, kde může sloužit jako vektor. Díky této skutenosti došlo v uplynulých letech k šíření hmyzem přenosných onemocnění postihujících lidi i domácí zvířata na území Evropy, s dopadem nejen na zdraví, ale také ekologickými a ekonomickými důsledky. Jedná se například o komáry přenášený virus západonilské horečky infekční pro lidi i zvířata, nebo dvě zoonózy postihující zejména ovce, katarální horečka ovcí a virus Schmallenberg, přenášené tiplíky.

V rámci širších projektů byly na území České republiky zpracovány dvě epidemiologické a entomologické studie cílené na komáry a tiplíky jako možné přenašeče viru západonilské horečky, katarální horečky ovcí či viru Schmalenberg Díky tomu byly na našem území zaznamenány nové druhy komárů i tiplíků, byla prokázána přítomnost viru západonilské horečky v komárech a také byl nalezen nový druh trypanosomy, *Trypanosoma culicavium*. Kromě faunistických studií a detekce patogenů v krevsajícím hmyzu byly studovány i hostitelské preference komárů, které úzce souvisí s přenosem patogenů mezi hostiteli, jelikož ovlivňují druhové spektrum možných hostitelů.

Introduction

Insects are the largest and most diverse class of animals, and among them are many species with some impact on humans and their activities, for better or worse. Since the late 19th century it's been known that bloodsucking insects can serve as vectors of organisms causing infectious diseases. Arthropod-borne diseases infect millions of people annually over the word; malaria, trypanosomiasis, leishmaniasis, filariasis, and dengue or yellow fever are all mosquito-borne diseases causing thousands of deaths per year. Seven of the top ten most important infectious diseases according to the World Health Organization are transmitted by insects such as mosquitoes, sand flies, black flies or kissing bugs. In addition to disease transmission, insects can cause hypersensitive reactions and allergies. Vector-borne diseases and allergies affect not only human health, but also livestock and wildlife, causing grave economic losses. In addition, insects can also serve as hosts for less important parasitic organisms, such as monoxenous trypanosomatids.

As the climate changes, the distribution and abundance of arthropods, including bloodsucking insects, can be affected. The emergence of new vector-borne diseases in Europe is likely to be amongst the most important effects of these changes. The spread of several arboviruses and global climate change suggest that both phenomena might be linked to each other. In recent years, several vector-borne diseases affecting both humans and domestic animals have re-emerged and spread in Europe with major consequences for health, ecology, and also for economics. Among them is the mosquito-borne West Nile virus, which also affects humans, and two zoonosis, the Bluetongue virus or Schmallenberg virus, transmitted by biting midges.

This PhD thesis focuses on two closely related groups of bloodsucking insects (mosquitoes and biting midges) as potential vectors of infectious diseases. The study brings new insights about mosquitoes and biting midge species found in the Czech Republic, where possible vectors of West Nile virus, Bluetongue or Schmallenberg virus are included. New mosquito and biting midge species were added to the check-lists of the Czech Republic. Presence of West Nile virus was found in mosquitoes as well as a new trypanosome species, *Trypanosoma culicavium*. Beside faunistic studies and screening for pathogen presence in insects, we also studied feeding preferences of mosquitoes, which strongly correlate with parasite transmission as they influence the spectrum of host contact.

Mosquitoes

Mosquitoes are one of the most studied groups of insects worldwide, as they are known to be vectors of many grave infectious diseases like malaria, yellow fever or dengue. In recent decades, several vector-borne diseases affecting human, livestock and also wild animals, have emerged in Europe (Halouzka and Hubálek 1996, Hubálek 2008). Some are exotic pathogens that have been introduced into new regions, and others are endemic species that have increased in incidence or the infection was developed for the first time at local human populations. Of particular interest are zoonoses that are hidden in wildlife populations but also affect livestock or eventually people who have been bitten by infected vectors. West Nile virus and Chikungunya virus are among the best explored zoonotic infections to have emerged in the past decades and show how expanding and dangerous can be in new regions (Kilpatrick and Randolph 2012).

Faunistics

Several faunistic studies were undertaken within the Czech and Slovak Republics. The most complete study of mosquito fauna of the former Czechoslovakia was published in 1958 (Kramář 1958) then some local studies were done (Minář and Halgoš 1997, Országh et al. 2006, Dvořák 2012). According to the older studies, 42 mosquito species have been recorded in our territory, 37 in Bohemia and 37 in Moravia (Országh et al. 2006). Some of the listed species were considered rare in Bohemia, for example *Culex modestus* or *Coquiletidia richiardii*. Most of the local studies of mosquito fauna were done in south Moravia, the warmest locality of our territory where the occurrence of mosquitoes has traditionally drawn much attention. Two important studies were done in south Bohemia, one at Lipno dam where 18 species were recorded (Minář 1975) and a study describing mosquito fauna of the Třeboň basin (Rettich et al. 1978) with 30 species detected. One of the most complex studies excluding south Bohemia and Moravia was linked to the massive floods in central Bohemia, where only ten species with two predominating *Ochlerotatus sticticus* and *Aedes vexans*, known as calamities species, were found (Rettich 2004). Other studies by this author were oriented around the Labe river, and were richer in species – Poděbrady 22 species (Rettich 1973), Hradec Králové 16 species (Rettich 1979) and Mělník 17 species (Rettich 1982). Regular trapping of females has been extensive only in two studies in south Moravia in the last few years, and 22 species of mosquitoes were trapped, including *Uranotaenia unguiculata* and *Anopheles hyrcanus* (Šebesta et al. 2009, Šebesta et al. 2010). The most recent study from the Lower Dyje basin confirmed 30 species from six mosquito genera present in our territory, again with the same two most frequent species *Ochlerotatus sticticus* and *Aedes vexans* (Šebesta, et al. 2012).

Tab. 1 Current list of mosquito species detected in the Czech Republic (simplified)

CULICINAE	*punctor*	*fumipennis*
Aedes	*refiki*	*glaphyroptera*
annulipes	*riparius*	*longiareolata*
cantans	*rossicus*	*morsitans*
caspius	*rusticus*	*ochroptera*
cataphylla	*sticticus*	*subochrea*
cinereus	*vexans*	***Coquillettidia***
communis	***Culex***	*richiardii*
dianteus	*hortensis*	***Uranotaenia***
dorsalis	*martinii*	*unguiculata*
excrucians	*pipiens molestus*	ANOPHELINAE
flavescens	*pipiens pipiens*	***Anopheles***
geniculata	*territans*	*atroparvus*
intrudens	*theileri*	*claviger*
leucomelas	*torrentium*	*hyrcanus*
nigrinus	***Culiseta***	*maculipennis*
pulcritarsis	*alaskaensis*	*messae*
pullatus	*annulata*	*plumbeus*

In Europe, 101 mosquito species have been recorded from eight genera (Snow and Ramsdale 2003). However, species composition is not stable, and it is evident that climate changes in recent years could enhance the spreading of thermophilic species into new localities (Reiter 2001). Recent records of Mediterranean species of mosquitoes in the former Czechoslovakia *Culex martinii* (Vaňhara 1986, Vaňhara and Rettich 1998) or from Slovak territory as *Culex theilerii* (Halgoš and Petrus 1996) and *Anopheles hyrcanus* (Halgos and Benková 2004) and the Czech Republic *Uranotaenia unguiculata* (Minář and Halgoš 1997) support this theory. Later on in 2005, we found *Anopheles hyrcanus* for the first time in Moravia (Votýpka et al.

2008). Our discovery was confirmed by a study on the same locality in 2008, where *Anopheles hyrcanus* was recorded as a constantly present species reaching a prevalence of more than 4 % (Šebesta et al. 2009). In 2010, *Anopheles hyrcanus* was also detected for the first time in Austria (Lebl et al. 2013). This mosquito species is a member of the *Anopheles hyrcanus* group, consisting of about 30 species in the Old World, including vectors of Malaria and having a large Palearctic distribution covering the southern part of Europe, the Mediterranean and central Asia (Ramsdale and Snow 2000). *Anopheles hyrcanus* does not transmit malaria in our country, but has been shown to be a vector of Sindbis and Ťahyňa virus and is a suspected vector of dirofilariasis (Hubálek 2008, Hubalek et al. 2014).

There is not only the introduction of new species, but also the expansion of earlier rare populations of thermophilic species which have become common as a vector of West nile virus *Culex modestus* in the Czech Republic (Votýpka et al. 2008). As the region of South Moravia is known to have a mild climate, the most convenient habitat for mosquito breeding within the Czech Republic (Šebesta et al. 2012), it can be also favourable for local mosquitoes as potential vectors of pathogens, from which the Ťahyňa, Batai, Lednice, and West Nile viruses have been recorded (Danielová et al. 1972, Hubálek et al. 1998, Juricová et al. 2009, Rádrová et al. 2013). Based on previous studies, we can assume that this locality can be a "gateway" for new species of mosquitoes. Moreover, in this region, larvae of the invasive Asian tiger mosquito species *Aedes albopictus* were discovered (Šebesta et al. 2012). It is well known as an important vector of human pathogens such as Chikungunya and dengue viruses as well as filarial nematodes represented by *Dirofilaria* (Medlock et al. 2012). Migration of thermophilic species to the north is not only a curious event, but also a serious problem due to the broad range of infectious diseases transmitted by some of them, particularly mosquitoes.

West Nile virus

West Nile fever caused by the West Nile virus family Flaviviridae, is a mosquito borne disease which is pathogenic for some bird species, horses and humans with several symptoms from light flu to serious neurological problems, though infections are most often asymptomatic (Zeller and Schuffenecker 2004). Although as a disease it's probably been present in Europe for a long time, the first indication of the presence of West Nile virus appeared in 1958, when specific antibodies were proved in two Albanians (Bardos et al. 1959). That WNV is an emerging and re-emerging disease can be demonstrated in the following cases; United States (Garmendia et al. 2001), France (Balenghien et al. 2006), Israel (Giladi et al. 2001) and many others (Petersen et al. 2001). WNV has been isolated in many areas of Africa, Europe, Asia, including the India subcontinent, as well as in North America since 1999. Outbreaks and sporadic cases of West Nile fever were reported generally in Mediterranean countries and in south-eastern Europe (Hubálek and Halouzka 1999, Zeller and Schuffenecker 2004). Human cases of West Nile fever also occurred in the Czech Republic in July 1997, after heavy rains caused extensive floods along the Morava River (Hubálek and Halouzka 1999). Phylogenetic analyses have revealed up to nine major lineages of WNV (Bakonyi et al. 2005, Bondre et al. 2007, Botha et al. 2008, Mackenzie and Williams 2009, Kemenesi et al. 2014) usually in accordance with geographical distribution of the virus. The flavivirus strain isolated in the Czech Republic from *Culex pipens* mosquitoes in 1997 was named as Rabensburg virus (RabV), but exhibited a close antigenic relationship to WNV as well as the complete nucleotide and putative amino acid sequence (Bakonyi et al. 2005). Strains from the same lineage were also found in our study conducted in south Moravia from 2004 – 2007 (Rádrová et al. 2013). Among the several mosquito species recorded in different European WNV outbreaks localities, *Culex pipiens*, *Cx. modestus*, and *Coquillettidia richiardii* are considered the main WNV vectors, based on their abundance, feeding behaviour, previous WNV isolations, and recent experimental transmission (Hubálek and Halouzka 1999, Savage et al. 1999, Balenghien et al. 2006). In our study, the Rabensburg virus, one of the lineages of West Nile virus *sensu lato* was detected in *Culex pipiens* and *Cx. modestus* mosquitoes (Rádrová et al. 2013). Later on in 2013, four strains of West Nile virus (WNV) lineage 2 were isolated from *Culex modestus* mosquitoes in south Moravia which were related to Austrian, Italian or Serbian isolates (Rudolf et al. 2014).

Feeding preferences

Transmission between vertebrate hosts is achieved by the blood-feeding habit of the mosquitoes, which allow the pathogen to successfully become established in and be transmitted by their arthropod hosts. Apart from the presence of a reservoir host species in the locality, vector behaviour and vector capacity, feeding preferences and spatial distribution of host-seeking mosquitoes are both crutial aspects of pathogen transmission. These characteristics influence the spectrum of host contacts and consequently the role of mosquitoes as vectors of wild and domestic animal pathogens, including possible transmission of pathogens to humans. Many mosquitoes manifest an opportunistic host choice, but some species are really host specific. Those species that express strong and constant host-selection behaviour are the most important vectors of infectious diseases, which suggests that this behaviour may have evolved parallel to parasite-host evolution (Takken and Verhulst 2013).

There are many approaches to studying feeding preferences – detection of source blood from blood fed females (for example the use of direct sequencing of a selected host blood gene), observing host selection using animal baited traps and other techniques. The second approach was undertaken in a study in France to determine potential vectors of West Nile virus in Camarque, France. Both *Culex* species in which West Nile virus was detected in the Czech Republic (Rádrová et al. 2013) have also been shown to be linked to WNV transmission: *Cx. modestus* in France (Mouchet et al. 1970) and *Cx. pipiens* in Israel (Goldblum et al. 1954), Romania (Savage et al. 1999), and the United States (Bernard et al.). These two species are strongly ornithophilic and good enzootic vector candidates in wet areas of Camargue. *Culex modestus* aggressively fed on people and horses and is a "bridge" vector candidate in wet areas of Camargue. *Cx. pipiens* (more ornitophilic than *Cx. modestus*) also fed on horses and acts as a "bridge" vector according to some authors (Kilpatrick et al. 2005). Use of animal baited traps in Sweden proved a high willingness of Nordic populations of *Cx. pipiens* to feed on birds (Jaenson 1990). A similar study was also performed in Israel (Braverman et al. 1991) where *Cx. pipiens* preferred mammalian hosts. With a different method, precipitation test, the same result was found in Egypt (Zimmerman et al. 1988) with 85 % of ungulates as a source of blood. In north-eastern Italy, 77 % of *Cx. pipiens* mosquitoes fed on a broad spectrum of birds, but some willingness to feed on mammals is clear (23 %) (Rizzoli et al. 2015), similar patterns were observed in Spain (Muñoz et al. 2012). In the USA *Cx. pipiens* was considered predominantly ornithophilic, but is willing to feed on mammals (Apperson et al. 2002, Hamer et al. 2009). Some studies also describe feeding on cold-blooded vertebrates like frogs, lizards or snakes (Apperson et al. 2002, Medlock et al. 2005). The mixed host preferences of the Czech *Cx.*

pipiens populations (Radrova et al. 2013) support the hypothesis of a north-south gradient of *Cx. pipiens* mosquito host preferences, ranging from strictly ornithophilic populations in the north to mammaliophilic populations in the south. Willingness to feed on both birds and mammals hosts contrasts with the previously reported strict ornithophily of central European *Cx. pipiens* populations (Kramář 1958, Rettich, personal communication) and we can speculate about the explanation; changes of feeding behaviour in our populations, or a shift of southern (ergo more mammaliophilic) mosquito populations to the north due to climatic changes.

Another species involved in West Nile virus transmission is *Culex modestus*. In studies describing mosquito fauna of our territory it's mentioned as rare (Kramář 1958, Vaňhara and Rettich 1998). Nevertheless, we have demonstrated the spread of this species throughout the Czech Republic in recent years (Votýpka et al. 2008). Little is known about the feeding behaviour of central European populations, however populations of other European regions are opportunistic and attack birds as well as mammals, including humans. In France, *Cx. modestus* fed on birds and also on humans and horses (Balenghien et al. 2006). Similarly in our study, *Cx. modestus* does not exhibit a preference for either rabbits (53 %) or Japanese quail (47 %) (Rádrová et al. 2013). On the contrary, a higher shift to ornitophilia was described in the Russian population and the same observation was made in south Moravia using sentinel hosts, *Cx. modestus* mosquitoes preferred ducks and chickens, while rabbits were ignored (Minar 1969). Both *Culex* species were observed to feed on both, birds and mammals with different proportions in different countries, and as West Nile virus was detected in both, they may be considered as potential WNV "bridge vectors" between birds and humans as well as main vectors in the sylvatic avian cycle (Platonov et al. 2008). Despite this, all findings should be tentative, as host preferences can often show a high degree of variability, caused mostly by environmental conditions when favourite host species are not available (Takken and Verhulst 2013).

Trypanosomatids

Family trypanosomatidae belong with the evolutionary primitive bodonids into Kinetoplastea (Lukeš et al. 2014), Euglenozoa, Excavata (Adl et al. 2012). Trypanosomatida are one of the most studied groups of protists. They have global distribution, and occur wherever their hosts are. Nowadays, it seems that some species have more global distribution and others are rather endemics (Votýpka et al. 2010, Votýpka, Klepetková, et al. 2012). Between characters unique to Trypanosomatida belongs one flagellum emerging from the flagellar pocket. Flagellum can be free, or adhere to a cell as an undulatory membrane. They possess a characteristic of an organelle called kinetoplast, a network of circular DNA inside a large mitochondrion (Baker 1976, Lukes et al. 2002). A huge diversity of morfotypes (amastigote, promastigote, ophistomastigote, trypomastigote, epimastigote, etc.) are also typical, and are characteristic for individual species (Vickerman 2000, Maslov et al. 2013). Different morphotypes also differ between particular stages in the life-cycle of trypanosomatids.

In between eukaryotic parasitic groups, trypanosomatids have quite a lot of species. According to their life-cycles, we can distinguish dixenous genera (*Trypanosoma*, *Leishmania*, *Phytomonas*) where invertebrate host serve as a vector, and genera monoxenous species (Maslov et al. 2013, Lukeš et al. 2014). Especially in monoxenous trypanosomatids, new species and genera are constantly described as *Sergeia podlipaevi* (Svobodová et al. 2007), *Wallaceinomonas* (Votýpka and Kostygov 2014, Yurchenko et al. 2014) *Kentomonas,* endosymbiont-harboring trypanosomatid from *Sarcophaga* (Votýpka and Kostygov 2014), *Blechomonas* from fleas (Votýpka et al. 2013) or *Paratrypanosoma confusum*, branching out with very high support at the base of the family Trypanosomatidae (Flegontov et al. 2013). In the group of dixenous trypanosomatids, the description of new genera is not so common, but it is possible to discover new species for example from bats (Grisard et al. 2003, Lima et al. 2012) or new genotypes/subspecies form tsetse flies (Votýpka, Rádrová et al., in press) .

The genus *Trypanosoma* is one of the most important, as several *Trypanosoma* species cause serious diseases of humans (Chagas disease, sleeping sickness) and domestic animals (nagana, surra, mal de caderas, dourine) (Mehlhorn and Aspöck 2008). On the contrary, infections in wild animals including bird hosts are in most cases asymptomatic (Baker 1976). Similarly as other members of the genus, avian trypanosomes have digenetic life cycles (Baker 1976, Votýpka and Svobodova 2004, Zídková et al. 2012). However, avian trypanosomes, their cycles and vectors are in general very little explored (Sehgal et al. 2001, Votýpka and

Svobodova 2004, Valkiūnas et al. 2011) even though they are surprisingly successful blood parasites and are widespread and prevalent in birds throughout the world (Bennett et al. 1982).

Avian trypanosomes are transmitted by a wide variety of blood-sucking arthropods belonging to the family Simuliidae, Culicidae, Ceratopogonidae and Hippoboscidae (Baker 1976, Linley 1985, Votýpka and Svobodova 2004, Votýpka, Szabová, et al. 2012, Zídková et al. 2012). In invertebrate vectors, trypanosomes multiply in the intestine and transmission is completed by ingestion of an infected insect or by contaminative transmission through feces (Votýpka and Svobodova 2004). It is also transmitted by predation among birds from the infected prey ingested by a raptor, it is assumed (Dirie et al. 1990).

Two obsolete approaches were applied in research of avian trypanosomes. Either every trypanosome from every single bird host was counted as a different species, or all trypanosomes from birds were included in the *Trypanosoma avium* complex. The result was a majority of provisional species names of avian trypanosomes and the necessity for a more accurate fylogenetic classification. In later phylogenetic studies avian trypanosomes form three complexes of species: *Trypanosoma avium*, *T. corvi* and *T. benetti* (Votýpka et al. 2004). The up-to-date study split avian trypanosomes into 11 separate lineages, three of them in accordance of previously described life-cycle data. The *T. corvi* lineage was formed by isolates from hippoboscid flies, songbirds and newly by raptors, the *T. culicavium* lineage, avian trypanosoma described during our study, was formed by mosquitoes and insectivorous songbirds, and isolates from black flies, songbirds and raptors were found in the *T.* cf. *avium* lineage (Zídková et al. 2012).

During our research on trypanosomatids, many species of monoxenous trypanosomatids were isolated from mosquitoes and biting midges. As the results are not published yet, they are not a part of this thesis.

Biting midges

Over the last decades, several emerging or re-emerging infection diseases transmitted by blood-sucking arthropods have been recorded in Central Europe (Hubálek et al. 1998, Votýpka et al. 2008). Biting midges of the genus *Culicoides* are frequently studied as an important subject in human and veterinary medicine and the interest was considerably increased in recent years in relation to outbreaks of their transmitted viral diseases.

Within the genus *Culicoides* (Diptera: Ceratopogonidae) 1.343 known species classified in 31 subgenera are included (Beckenbach and Borkent 2003). The adult *Culicoides* female are haematophagous parasites of a broad range of host organisms including humans, livestock and other mammals, amphibians or birds. Some of them are important vectors of pathogens responsible for several diseases with veterinary and public health importance, especially three OIE (World Organisation for Animal Health) notifiable diseases of ruminants and equines, respectively Bluetongue virus (BTV), Schmallenberg virus and African horse sickness (Meiswinkel and Paweska 2003, Carpenter et al. 2013, Elbers et al. 2013). Furthermore, they also transmit other viruses such as the Oropouche virus (Linley et al. 1983, Mellor et al. 2000) and Rift Valley fever virus detected in females caught in the wild (Jennings et al. 1982). In the USA, West Nile virus RNA was isolated from two *Culicoides* species (Sabio et al. 2006). In addition, in the Czech Republic two strains of Ťahyňa virus were isolated as the first report of arbovirus from biting midges in Europe (Halouzka et al. 1991). Biting midges are also vectors of filaria most notably *Mansonella* species, which can affect various species including humans (Linley 1985, Mellor et al. 2000, Carpenter et al. 2013). Despite the biting nuisance and the role as vectors of worldvide important arboviruses of livestock (Mellor et al. 2000), *Culicoides* have only rarely been implicated as the primary vectors of pathogen to or between humans, with the exception of the *Mansonella* filariae and Oropouche viruses. On top of pathogen transmission, *Culicoides* biting midges cause intense pruritic dermatitis of horses, commonly known as sweet itch (Anderson et al. 1991, Mullens et al. 2005).

The Schmallenberg virus and especially the Bluetongue virus have been in the spotlight for the last few years. The Bluetongue virus was previously considered an exotic disease with just a few sporadic offensives up to 1998. Later on, Europe has suffered a series of epizootic outbreaks of BTV with serious impact on animal health and economics. In August 2006 BTV appeared in the Netherlands, the first ever case of BTV infection in Western Europe (Anon 2006). During the following months, this outbreak spread to infect animals in Belgium, Germany, France and Luxembourg

(Wilson and Mellor 2009). What’s more, it was a serotype BTV-8 which hadn’t occurred earlier in Europe, demonstrating that the whole of Europe is at risk (Mertens et al. 2007). Until now, eight BTV serotypes have spread throughout Europe (Zientara and Sánchez-Vizcaíno 2013).

The vectors of bluetongue virus are some species of *Culicoides* biting midges, but only the Mediterranean species *Culicoides imicola* has long been considered the only vector (Baylis 2002, Mellor and Wittmann 2002, Goffredo et al. 2003). However, the distribution pattern of *C. imicola* did not always correspond either geographically or seasonally with virus circulation, so the search for local vectors was undertaken in many European countries. During the emergence of the BTV epidemic in Northern Europe, most EU countries started the monitoring of biting midges (e.g. Meiswinkel et al. 2008, Sehnal et al. 2008, Casati et al. 2009, Hörbrand and Geier 2009). The potential vectors of BTV in northern Europe belong to the *obsoletus* and *pulicaris* groups, namely *Culicoides obsoletus*, *C. scoticus*, *C. dewulfi*, *C. chiopterus* and *C. pulicaris*. Their vector potentials were determined either by viral isolation or virus detection by reverse transcriptase quantitative polymerase chain reaction (Caracappa et al. 2003, De Liberato et al. 2005, Savini et al. 2005, Meiswinkel et al. 2007, Dijkstra et al. 2008).

The majority of entomological studies about biting midges in our country go back a in the past, are limited in scope and did not specifically target the species in the surroundings of the animals whose health could potentially be impacted. The biting midge fauna was well described by Országh (Országh 1980) who enumerated 63 species of *Culicoides* in the area of the former Czechoslovakia. After this study, several papers appeared about diversity, biology and the abundance of biting midges or introducing of new species of *Culicoides*, but did not include all regions of our country. The most common and most applied methods in these studies were catching by light traps, Malaise traps and entomological nets, or even a network attached on the hood of a car. In 1997 the list of species was updated, ten species of biting midges was added. Nine of them were found during studies on the south Moravia (Knoz 1987, 1997, Knoz and Michálek 1987, Knoz and Vaňhara 1991), the last one from south Bohemia (Knoz 1997). In the end, 47 *Culicoides* species were detected in the territory of Bohemia and Moravia. After 2000, another two species were found in south Moravia (Tóthová et al. 2004, Tóthová et al. 2005). Afterwards, no new species were found during entomological studies in south and north Bohemia (Knoz et al. 2004, Knoz and Tóthová 2008). The last and the most up-to-date list of *Culicoides* species report 49 species for the lands comprising Bohemia and Moravia (Tóthová and Knoz 2009). For our neighbours and former part of Czechoslovakia, the Slovak Republic, 55 species of *Culicoides* were known (Tóthová and Knoz 2009) with *C. newsteadi*, *C. longicollis*, *C. puncticollis*, *C. cameroni*, *C. dispersus*, *C. zhogolevi*, *C. saevus*, and *C. slovacus* which are not listed for the Czech Republic. On the contrary, two species, *C. sphagnumensis* and *C. haranti* are present only on the territory of the Czech Republic, not recorded in Slovakia. In the last study, five new species were added to the list for Slovakia: *C. picturatus, C. gejgelensis*, *C. clastrieri*, *C. griseidorsum* and *C. odiatus* (Sarvašová et al. 2014). Three of these species, previously not found in the Czech Republic, *C. clastrieri, C. odiatus* and *C. saevus* were recorded for the first time during our biting midge survey (Rádrová et al. subm.).

Tab. 2 Current list of *Culicoides* biting midge species detected in the Czech Republic (* newly recorded species – Rádrová et al. submitted)

Culicoides	*grisescens*	*clastrieri* *	*pseudoheliophilus*
Avaritia	*impunctatus*	*comosioculatus*	*reconditus*
abchazicus	*pulicaris*	*duddingstoni*	*segnis*
chiopterus	*punctatus*	*dzhafarovi*	*semimaculatus*
dewulfi	*jurensis*	*fascipennis*	*shaklawensis*
obsoletus	**Monoculicoides**	*festivipennis*	*simulator*
scoticus	*nubeculosus*	*furcillatus*	*subfasciipennis*
Beltranmyia	*parroti*	*haranti*	*truncorum*
circumscriptus	*riethi*	*heliophilus*	*ustinovi*
manchuriensis	*stigma*	*kibunensis*	*vexans*
salinarius	**Oecacta**	*maritimus*	*vidourlensis*
sphagnumensis	*achrayi*	*minutissimus*	**Pontoculicoides**
Culicoides	*albicans*	*odiatus* *	*tauricus*
delta	*alazanicus*	*pallidicornis*	*saevus**
fagineus	*brunnicans*	*pictipennis*	

Aims of the thesis

- To describe mosquito species occurring in selected wetland localities of the Czech Republic.

- To study feeding preferences of selected mosquito species.

- Detection of transmitted pathogens in mosquitoes with focus on West Nile virus and trypanosomatids.

- To describe biting midges fauna attacking livestock and hoofed game in the frame of Bluetongue virus surveillance programme.

Publications

Votýpka J., Šeblová V., and Rádrová J. 2008. Spread of the West Nile virus vector *Culex modestus* and the potential malaria vector *Anopheles hyrcanus* in central Europe. Journal of Vector Ecology. 33: 269–277.

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Spread of the West Nile virus vector *Culex modestus* and the potential malaria vector *Anopheles hyrcanus* in central Europe

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ABSTRACT: Mosquito faunal studies were carried out in five separate wetland regions in the Czech Republic during 2004-2007, sampling with dry ice-baited and sentinel host-baited CDC traps. A total of 79,245 adults was identified, representing 23 mosquito species that belonged to the genera *Anopheles, Culiseta, Coquillettidia*, *Aedes*, and *Culex*. Our findings reveal that the mosquito fauna is enriched by new elements in the Mediterranean region. Historical and CDC trap data suggest that the newly-emerging potential malaria vector, *Anopheles hyrcanus,* has reached the northern limit of its distribution in the Czech Republic, and the important West Nile virus (WNV) vector, *Culex modestus,* has widened its distribution in the Czech Republic. No significant differences were observed in a total number of mosquitoes collected by traps baited with either the sentinel animals or with CO_2, although species abundance differed. A relatively higher proportion of *Cx. modestus* was collected in the sentinel-baited traps, while the proportion of *Cx. pipiens* was higher in the CO_2-baited traps. ***Journal of Vector Ecology*** **33 (2): 269-277. 2008.**

Keyword Index: Mosquitoes, WNV, Czech Republic, climate change.

INTRODUCTION

In recent years, several vector-borne diseases affecting both humans and domestic animals have re-emerged and spread in Europe with major health, ecological, socio-economical, and political consequences (Reiter 2001, Gubler 2002, Zell 2004, Rogers and Randolph 2006). Despite a temperate climate and high economic and hygiene standards, several mosquito-borne viruses appear to circulate in Central Europe (Hubálek and Halouzka 1996, 1999). Serological surveys and viral isolates from mosquitoes indicate that Sindbis (SINV), West Nile virus (WNV), Usutu virus (USUV), and partially Batai virus (BATV) are widespread and probably enzootic in many countries of the region (Hubálek and Halouzka 1996, Gratz 2004, Hubálek et al. 2005). WNV has emerged and re-emerged as has also been demonstrated in the United States (Garmendia et al. 2001). During the past 40 years, human and equine outbreaks of WNV were reported from many European countries, and human cases of West Nile fever also occurred in the Czech Republic (southern Moravia) in July 1997, after heavy rains caused extensive flooding along the Morava River (Hubálek and Halouzka 1999). Based on the abundance, feeding behavior, previous WNV isolations, and recent experimental transmission, several mosquito species were implicated as the main WNV vectors in the European WNV outbreak, including *Culex pipiens*, *Cx. modestus*, and *Coquillettidia richiardii* (Hubálek and Halouzka 1999, Balenghien et al. 2006).

Mosquito abundance is monitored world-wide and these insects serve as a suitable group for studying changes caused by trends in environmental conditions (e.g., Hemmerter et al. 2007). These types of studies were undertaken by several authors within the Czech and Slovak Republics. Since 1958, mosquito distribution was determined in just a few localities (Kramář 1958, Minář and Halgoš 1997, Országh et al. 2006). So far, 42 mosquito species have been recorded in the Czech Republic (37 in Bohemia and 37 in Moravia) (Országh et al. 2006), with some of them considered as rare (e.g., vectors of WNV – *Cx. modestus* and *Cq. richiardii* in Bohemia). However, species composition is not stable in time, and climate change during recent years resulted in several new records of Mediterranean mosquito species in Slovak territory, including *Culex theileri* (Halgoš and Petrus 1996) and *Anopheles hyrcanus* (Halgoš and Benková 2004). Both of these species are currently absent in the Czech Republic. Similarly in southern Moravia, two new thermophilic species were recorded during the last three decades: *Culex martinii* and *Uranotaenia unguiculata* (Vaňhara 1981; Minář and Halgoš 1997).

Our mosquito surveillance program focused on the distribution, vector capacity, and feeding behavior of mosquitoes with the following goals: 1) to monitor mosquito populations and changes in species composition over time at several localities in the Czech Republic (Bohemia and Moravia), 2) to detect feeding preferences and behavior as well as spatial distribution of the mosquito species involved in WNV transmission, and 3) to test the mosquitoes and wild and domestic birds for WNV in order to identify possible disease foci. In the present article, we provide a report on the first objective.

MATERIALS AND METHODS

Our study was conducted in 2004-2007 to determine mosquito diversity at five separate wetland areas in the Czech Republic. Sampling was initiated at the end of June and terminated in August. In one locality (Blatná), traps were placed weekly from the beginning of April and continuing through October.

We studied the occurrence of mosquitoes around fishponds in various localities in the Czech Republic from 2004 to 2007. The wet fishpond areas of southern Bohemia and the large river basin in southern Moravia host rich populations of migratory and resident birds. CDC traps were placed in five different regions (four in Bohemia and one in Moravia), represented by seven distant localities (four in southern and one in western, central, and eastern Bohemia, and southern Moravia) and 26 sites selected according to local conditions and distribution of water sources across the landscape (Table 1, Figure 1). Four localities were situated in southern Bohemia and included seven fishponds sites around Blatná city (Zadní Topič – 49°25’N, 13°53’E, 432 MSL; Kaneček – 49°26’N, 13°53’E, 445 MSL; Podskalák – 49°26’N, 13°52’E, 447 MSL; Lhotka – 49°24’N, 13°51’E, 508 MSL; Žabinec – 49°24’N, 13°48’E, 487 MSL; Hříbárna – 49°24’N, 13°52’E, 483 MSL; Lomnice – 49°25’N, 13°52’E, 437 MSL), four fishponds sites around Česke Budějovice city (Černíš – 49°0’N, 14°24’E, 384 MSL; Vrbenský – 49°0’N, 14°26’E, 391 MSL; Zadní Topole – 49°3’N, 14°22’E, 386 MSL), fishpond Blatec (49°6’N, 14°18’E, 393 MSL), and a protected landscape area, Řežabinec (49°15’N, 14°5’E, 380 MSL). In southern Moravia, traps were situated at five fishpond sites near Mikulov city (Nestyt – 48°46’N, 16°43’E, 178 MSL; Křivé – 48°50’N, 16°42’E, 290 MSL; Šibeník – 48°47’N, 16°37’E, 196 MSL; Nový – 48°47´N, 16°40’E, 192 MSL; Milovický forest – 48°50’N, 16°42’E, 290 MSL). As far as the weather permitted, traps were placed daily during testing periods, and in the majority of sites both types of CDC traps (CO_2-baited and sentinel–baited) were used (Table 1).

In addition to the above-mentioned places, we studied mosquito occurrence around fishponds in western Bohemia at four sites (Bezděkovský – 49°45’N, 12°42’E, 511 MSL; Borský – 49°45’N, 12°43’E, 495 MSL; Modrý – 49°44’N, 12°44’E, 481 MSL; Regent – 49°54’N, 12°44’E, 535 MSL), in central Bohemia around fishponds within Prague (V Pískovně – 50°5’N, 14°34’E, 240 MSL; Litožnice – 50°4’N, 14°36’E, 256 MSL), and in eastern Bohemia at three sites (Nový – 49°49’N, 15°27’E, 400 MSL; Rousínov – 49°50’N, 15°27’E, 346 MSL; Běstvina – 49°49’N, 15°36’E, 357 MSL). Most of these sites in western, central, and eastern Bohemia were visited only once or twice, and only CO_2–baited CDC traps were used. All of these sites were monitored to confirm the occurrence of *Cx. modestus* and other mosquito species that had yet to be identified. For that reason, all data presented in this study are only related to catches from southern Bohemia and southern Moravia, with the only exception related to the distribution of *Culex modestus* throughout the Czech Republic.

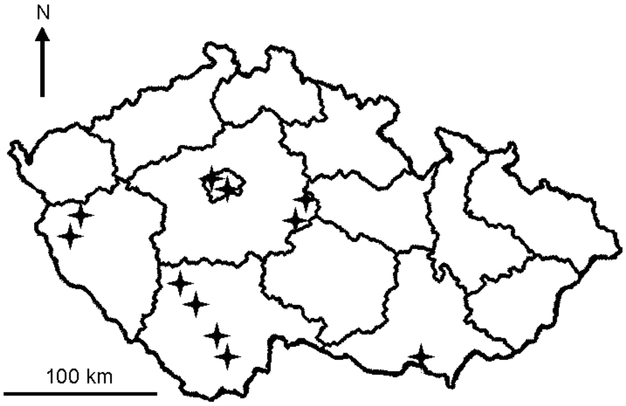

Figure 1. Map of all collecting localities in the Czech Republic where *Culex modestus* were found.

Dry ice–baited or sentinel host–baited CDC miniature light traps (Models 512 and 1012, John W. Hock Company, Gainesville, FL) without bulbs were placed near fishponds, open water pools overgrown with vegetation, and several other natural and artificial aquatic habitats in a variety of land-use areas. CDC traps baited with dry ice were set up in all studied sites for two to five consecutive nights. They were set out before dusk, and were picked up next morning. Chickens and Japanese quail were used as sentinel birds, while rabbits and guinea pigs were sentinel mammals. Animal hosts were placed in cages just before transporting them to field sites and returned within an hour after removing the insect traps the next morning. Birds had access to food and water during insect trapping. They were marked with colored tape on their legs and rotated among the trap locations. Because two CDC light traps were placed near each animal-baited cage, catches from both traps were pooled and considered as the catch of one trap. Animal-baited cages consisted of a double cage (inner cage: 50×40×30 cm, outer cage: 60×50×35 cm) with a Plexiglas® roof to allow visual orientation of vectors and to protect the birds from rain. In total, 323 trap nights were performed for dry ice–baited and 62 for sentinel host–baited traps (Table 1). Except for the Blatná locality, traps were run for a total of 95 nights (eight nights between 2 July and 30 July 2004; 30 nights between 4 July and 20 September 2005; 40 nights between 5 June and 30 September 2006; 17 nights between 24 April and 11 August 2007).

Mosquitoes were killed with CO_2 and transported to the laboratory on dry ice. Specimens were sorted under a stereomicroscope and the numbers of unengorged and engorged females and males were recorded. They were identified to species and stored at -70° C for further investigations. Females separated by species were placed in pools of one to 50 specimens for subsequent testing for WNV by using a specific polymerase chain reaction (PCR) assay. These samples were stored at -70° C until tested. Blood meals of engorged females were extracted and preserved on filter papers and the source of blood was detected by sequencing. Results from the PCR tests will be reported separately. Adults were identified using morphological characters under a stereomicroscope. Males

Table 1. Mosquitoes collected at 26 trap sites separated by regions, localities, years, and type of CDC trap bait. T/N – Number of trap-nights per site; Mean – Mean number of mosquitoes per one trap-night ± SE.

		2004		2005				2006				2007	
		CO_2		CO_2		SENTINEL		CO_2		SENTINEL		CO_2	
Region	Locality	T/N	Mean ± SE	T/N	Mean ± SE	T/N	Mean ± SE	T/N	Mean ± SE	T/N	Mean ± SE	T/N	Mean ± SE
Southern B.	Blatná			21	75.8 ± 19.2			29	110.4 ± 33.1			17	47.6 ± 13.2
	Č. Budějovice			14	30.9 ± 6.6	6	13.5 ± 2.7	16	123.6 ± 13.5	16	36.1 ± 4.9		
	Blatec	5	175.2±68.2	6	26.7 ± 6.9	2	9.3 ± 5.7	19	306.0 ± 31.1	14	232.1 ± 98.4	14	36.3 ± 5.9
	Řežabinec	3	674.7±321.7	20	61.3 ± 8.7	6	35.8 ± 6.7	7	1033.6 ± 146.6	6	271.1 ± 61.9	12	11.9 ± 3.2
Southern M.	Mikulov			39	109.5 ± 17.3			32	541.0 ± 171.4	12	660.1 ± 140.1	33	129.3 ± 17.8
Western B.								6	75.4 ± 19.6			6	45.2 ± 6.8
Central B.	Prague			6	29.5 ± 9.1			6	82.6 ± 15.9				
Eastern B.				6	16.6 ± 5.6			6	168.5 ± 29.0				

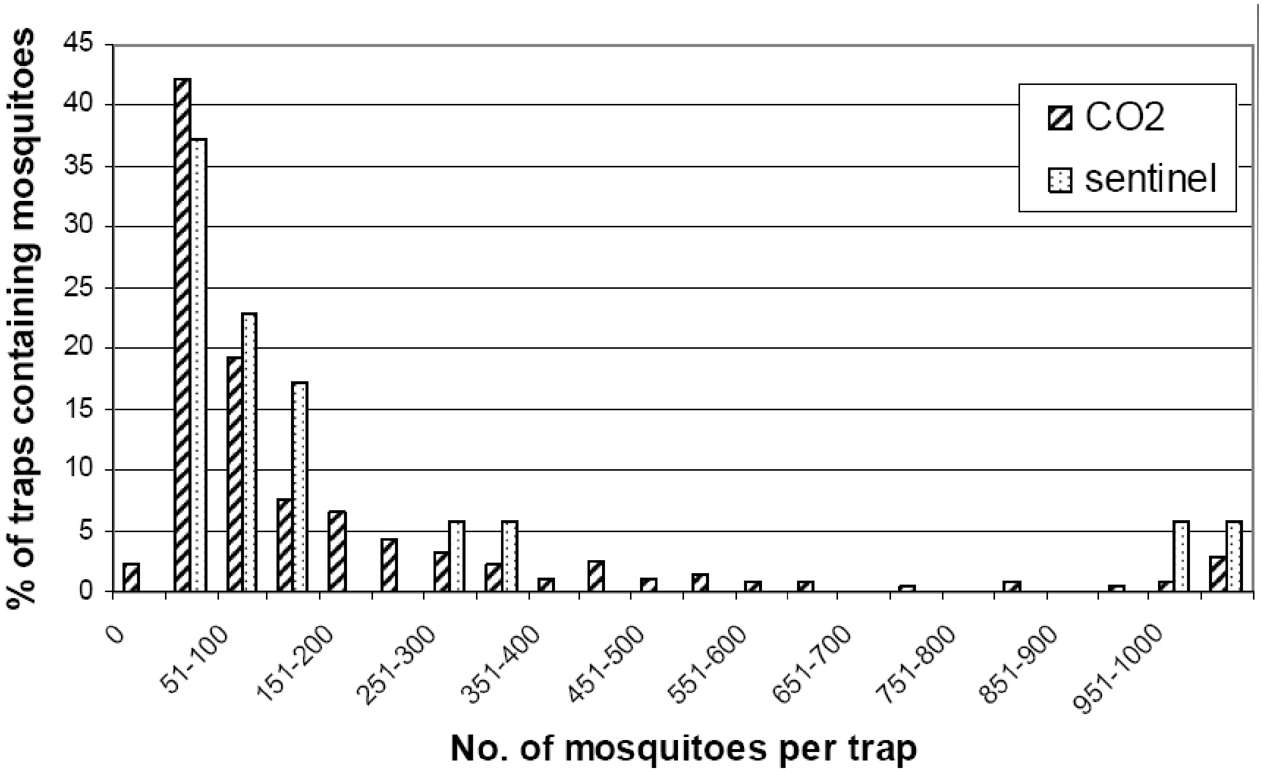

Figure 2. Number of mosquitoes collected in each of 323 CO_2-baited and 62 sentinel-baited trap nights over the study.

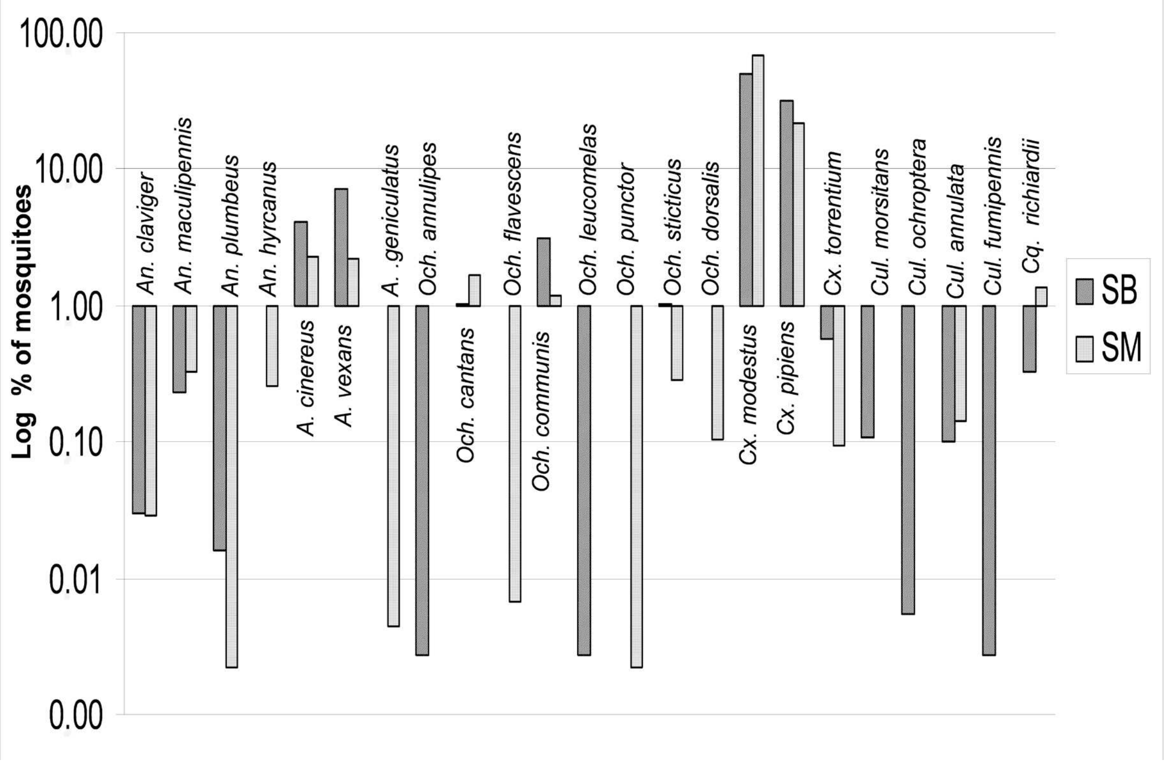

Figure 3. Relative proportion of all mosquito species captured in fishponds sites of southern Bohemia (SB) and southern Moravia (SM) during 2004–2007.

Table 2. List of mosquitoes collected near fishponds in the Czech Republic (2004–2007) separated by regions (SB – southern Bohemia, SM – southern Moravia) and type of CDC trap bait (CO_2 vs sentinel).

Species	Southern Bohemia		Southern Moravia	
	CO2	SENTINEL	CO2	SENTINEL
	No. mosquitoes (% of total)	No. mosquitoes (% of total)	No. mosquitoes (% of total)	No. mosquitoes (% of total)
Anopheles claviger	8 (0.03)	3 (0.04)	13 (0.05)	
Anopheles maculipennis	82 (0.32)	4 (0.06)	145 (0.56)	1 (0.00)
Anopheles plumbeus	6 (0.02)		1 (0.00)	
Anopheles hyrcanus			92 (0.36)	15 (0.07)
Aedes cinereus	1,054 (4.06)	559 (7.83)	871 (3.37)	139 (0.69)
Aedes vexans	2,237 (8.61)	340 (4.76)	926 (3.58)	50 (0.25)
Aedes geniculatus				1 (0.00)
Ochlerotatus annulipes	1 (0.00)			
Ochlerotatus cantans	370 (1.42)	5 (0.07)	754 (2.92)	7 (0.03)
Ochlerotatus flavescens			3 (0.01)	
Ochlerotatus communis	1,151 (4.43)	18 (0.25)	534 (2.07)	
Ochlerotatus leucomelas		1 (0.01)		
Ochlerotatus punctor				1 (0.00)
Ochlerotatus sticticus	320 (1.23)	58 (0.81)	122 (0.47)	6 (0.03)
Ochlerotatus dorsalis			47 (0.18)	
Culex modestus	12,253 (47.16)	3,809 (53.25)	14,005 (57.18)	16,286 (80.34)
Culex pipiens	8,116 (31.23)	2,323 (32.54)	7,634 (29.53)	3,750 (18.50)
Culex torrentium	214 (0.82)		42 (0.16)	
Culiseta morsitans	39 (0.15)	1 (0.01)		
Culiseta ochroptera	2 (0.01)			
Culiseta annulata	28 (0.11)	1 (0.01)	51 (0.20)	14 (0.07)
Culiseta fumipennis	1 (0.00)			
Coquillettidia richiardii	102 (0.39)	17 (0.24)	610 (2.36)	2 (0.01)
Total	25 984	7 139	25 850	20 272

were identified by observing their hypopygia on microscope slides in CMCP-10 mounting medium. Identifications were made with keys contained in Kramář (1958) as well as comparisons with a synoptic specimen collection at the Department of Parasitology, Charles University, Prague.

The absolute and relative effectiveness of two trapping methods (CO_2 vs sentinel animal) was evaluated by an analysis of variance (Statistica v. 6.0, main effect ANOVA) for all species together as well as for each of the species individually with respect to collection locality and season. As two CDC light traps were placed near each animal-baited cage, computation of trap effectiveness and selectiveness was achieved by analyzing the mean numbers of female mosquitoes collected per one trap.

RESULTS

We collected 79,245 mosquitoes during 385 trap-nights. Numbers of traps, mean numbers of mosquitoes in all regions, and numbers of each mosquito species collected by both type of traps (CO_2 and sentinel) in two well-studied regions (southern Bohemia and southern Moravia) are presented in Tables 1 and 2. A total of 51,834 mosquitoes from dry ice–baited CDC traps, representing 23 mosquito species belonging to five genera (*Anopheles, Culex, Culiseta, Coquillettidia*, and *Aedes*) and 27,411 mosquitoes from sentinel host–baited traps were collected (Table 2). The mean number of mosquitoes collected in each trap ranged from nine per trap night (sentinel trap) to a maximum of 1,033 per trap night (CO_2 trap) (Table 1). The number of mosquitoes collected in a single trap over one night ranged from 0 to 4,375 and 1,343 in CO_2 and sentinel–baited traps, respectively. Among all the traps, 1.4% (six) contained no

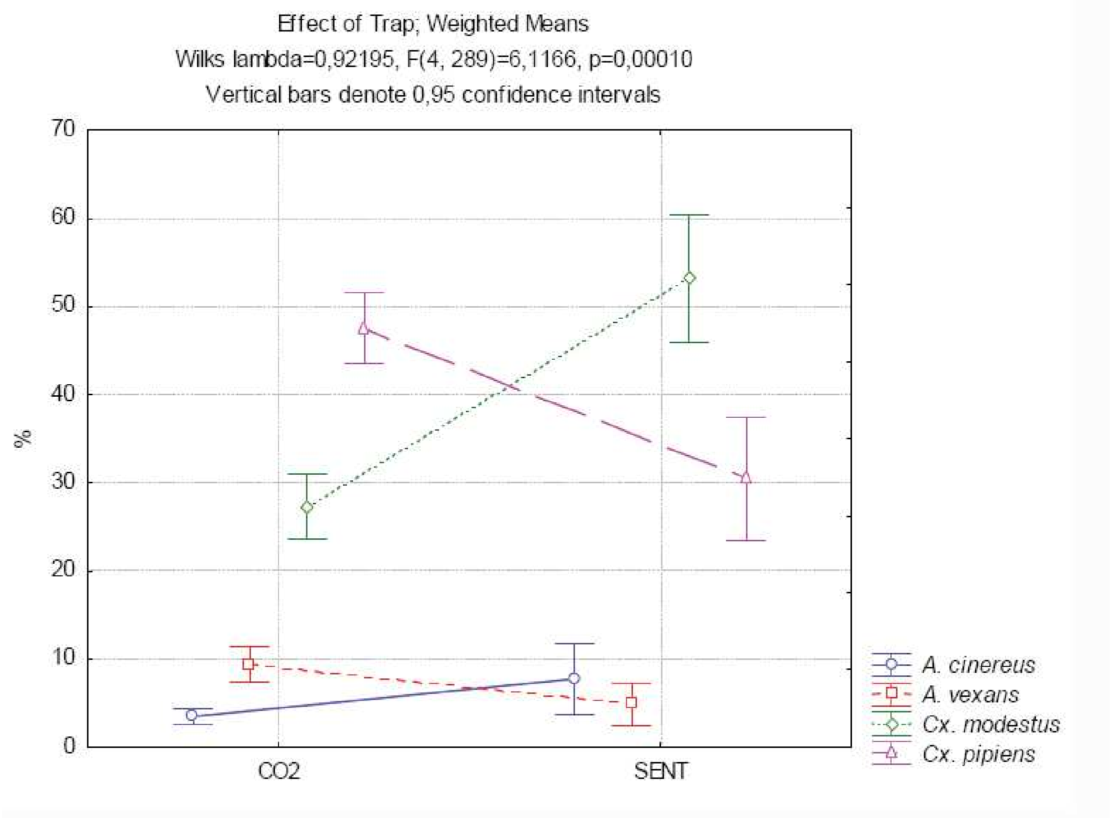

Figure 4. Relative proportion of *Cx. modestus* and *Cx. pipiens* according to trapping methods (CO2- baited vs sentinel-baited traps) in localities where both types of traps were set up together in 2005–2006.

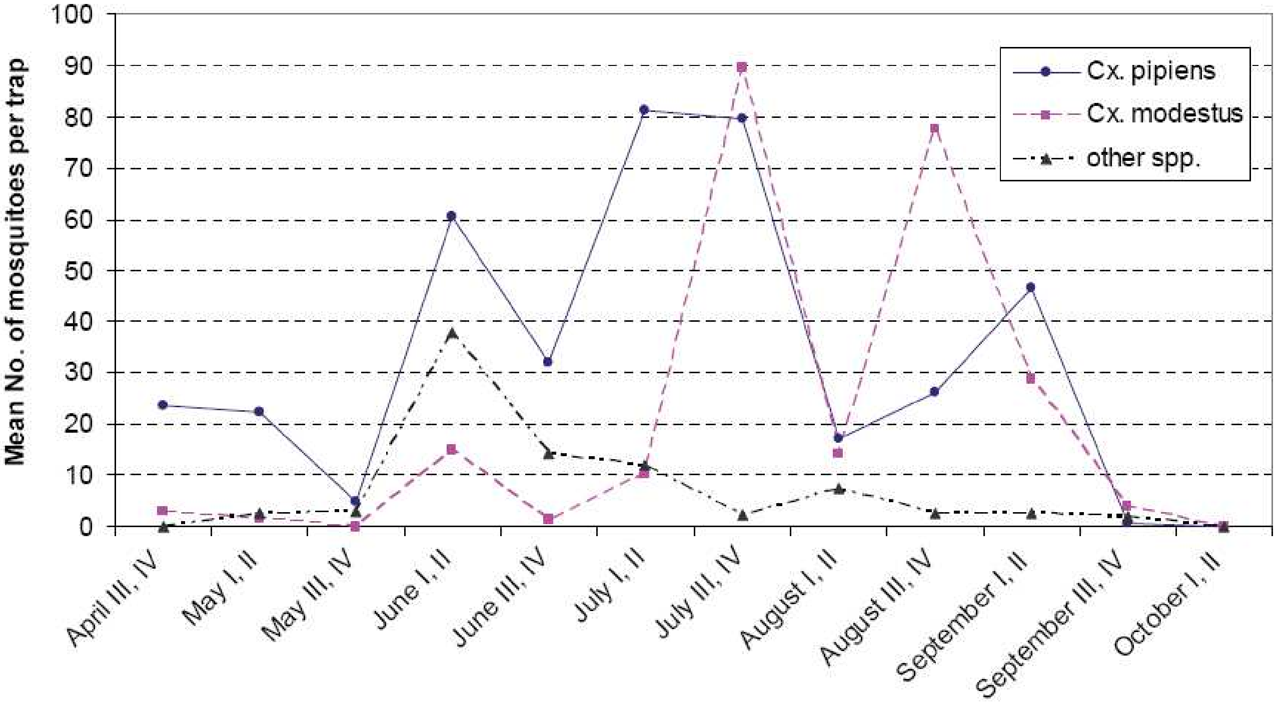

Figure 5. Temporal distribution of mosquitoes around seven fishponds in Blatná. Data represent collections made from April to October in 2005–2007.

mosquitoes at all and 37.6% (157) of the traps contained more than 100 (Figure 2).

Mosquitoes in the genera *Anopheles*, *Culex*, *Culiseta*, and *Coquillettidia* were identified to species, whereas *Aedes* were identified to species or species complex (e.g., *Aedes communis*). In 2004, 2,935 mosquitoes were collected over the course of eight trap nights. The most abundant species were *Cx. modestus* (62.3%), *Cx. pipiens* (14.4%), and *Ae. vexans* (12.3%). In 2005, 8,307 mosquitoes were collected over the course of 126 trap nights and the most abundant species were *Cx. pipiens* (53.7%), *Cx. modestus* (33.1%), and *Aedes vexans* (6.8%). In 2006, 62,276 mosquitoes were collected over the course of 169 trap nights and the most abundant species were *Cx. modestus* (65.4%), *Cx. pipiens* (21.6%), and *Ae. vexans* (3.6%). In 2007, 5,727 mosquitoes were collected over the course of 82 trap nights. The most abundant species were *Cx. pipiens* (61.3%), *Cx. modestus* (18.8%), and *Coquillettidia richiardii* (9.8%). The total mosquito collection data for all species captured in CO_2 and sentinel-baited-traps located in the wetlands areas of southern Bohemia and southern Moravia for all the years together are summarized in Table 2. Differences in distribution of all mosquito species between southern Bohemia and southern Moravia are presented in Figure 3.

The dominant species collected using both type of CDC traps during 2004–2007 in southern Bohemia were *Cx. modestus* (48.4%), *Cx. pipiens* (31.4%), and *Aedes vexans* (7.8%). The most common species collected in southern Moravia were *Cx. modestus* (65.3%), *Cx. pipiens* (24.6%), and *Aedes vexans* (2.2%). Among all mosquito species, *Cx. modestus* was the most dominant (58.5%), followed by *Cx. pipiens* (27.5%) and *Ae. vexans* (4.5%). With reference to genera, *Culex* spp. comprised 86.4%, *Aedes* spp. 12.1%, *Cq. richiardii* 0.9%, *Anopheles* 0.5%, and *Culiseta* spp. 0.2% of the mosquito fauna.

Occurrence of dominant species was similar in both well-studied regions, but the composition of less abundant species significantly differed (Figure 3). *Culiseta fumipennis*, *Culiseta ochroptera*, and *Culiseta morsitans* were captured only in southern Bohemia, whereas more prevalent *Anopheles hyrcanus* and *Aedes dorsalis* and a few specimens of *Aedes punctor, Aedes flavescens*, and *Aedes geniculatus* occurred only in southern Moravia. *An. hyrcanus* was repeatedly found during 2005–2007 at all studied sites in southern Moravia. Traps from western, central, and eastern Bohemia were specifically analyzed for the presence of *Cx. modestus*. This species was found at all studied sites (Figure 1) in relative high abundance, from 8% (western Bohemia) to 85% (eastern Bohemia).

To compare relative effectiveness and species selectiveness of both trapping methods (CO_2 vs sentinel), the average of the relative abundances of each species was evaluated with respect to the trapping localities (only localities of southern Bohemia and Moravia where both types of traps were set up together) and seasons (2005 and 2006). The effectiveness of CO_2-baited traps (effect of season and locality were controlled) was significantly higher ($F_{(1, 342)} = 6.8942$, $p<0.01$). Hence, relative proportions of appropriate mosquito species were used for further analyses. Four of the most abundant species collected over all tested localities during 2005–2006 were *Cx. modestus* (average of relative abundance was 31.1% and 54.4%), *Cx. pipiens* (45.5% and 31.4%), *Ae. vexans* (6.5% and 5.4%), and *Ae. cinereus* (4.1% and 6.8%) in accordance with CO_2-baited and sentinel-baited traps, respectively. The difference in the percentage proportion of these four species was highly significant ($F_{(4, 289)} = 6.1166$, $p<0.001$), and the relative abundance of the two most dominant species (*Cx. pipiens* and *Cx. modestus*) is presented in Figure 4.

The temporal abundance of mosquitoes collected by CO_2-baited traps in several sites within Blatná is presented in Figure 5 (combined results from 2005–2007). The numbers of mosquito species (*Cx. modestus* and *Cx. pipiens*) were in both cases low in April and May, started increasing in June, were highest from July until August, and decreased rapidly in late September. The first specimens of *Cx. pipiens* and *Cx. modestus* were observed on April 27th (in 2007) and the last was caught on September 24th (in 2006).

DISCUSSION

One of the most important factors limiting mosquito distribution is temperature, and a change of climate could influence mosquito populations in several ways, as has been demonstrated in America and southern Europe. On the other hand, only a few studies have focused on this topic in central Europe (Halgoš and Petrus 1996, Olejníček et al. 2004). The Czech Republic was affected by several catastrophic floods (1997 and 2002) that significantly influenced species composition, as well as the occurrence of mosquito-borne diseases (Olejníček et al. 2004, Rettich 2004, Hubálek et al. 1998, 2005), e.g., WNV in which transmission from birds to mammalian hosts occurred due to bridge vectors like *Cx. pipiens* (form *molestus*), *Cx. modestus*, and *Cq. richiardii*.

These European primary vectors of WNV have already been recorded from the Czech Republic, but only *Cx. pipiens* are repeatedly reported to be abundant, whereas *Cq. richiardii* is mentioned as a rare species occurring in lowlands. Our data unambiguously confirmed this mention, even though in some sites the density of *Cq. richiardii* was relatively high (up to 22.1%, 2007, southern Moravia, Mušlov), particularly in 2007, most likely due to the mild winter temperatures in 2006 and 2007. Despite high anthropophily, the low density of *Cq. richiardii* in both parts of the Czech Republic does not justify involving this species as a primary vector of WNV in central Europe.

The Mediterranean thermophilic species *Cx.* (*Barraudius*) *modestus* was reported to be common in Slovakia (Minář and Halgoš 1997, Jalili et al. 2000), medium-abundant in Moravia (Minář 1969, Vaňhara and Rettich 1998, Minář et al. 2001), and rare to very rare in Bohemia. In the last comprehensive monograph focused on mosquito fauna in the Czech Republic (Kramář 1958), only two specimens are mentioned (Velký Tisí fishpond in southern Bohemia in 1954). A later study (Rettich et al. 1978) described only two more localities for *Cx. modestus*

(Majdalena and Lomnice nad Lužnicí, both in southern Bohemia) and authors mentioned this species as "rare." Although a number of studies that focused on the mosquito fauna of several localities within Bohemia were published, no additional specimens were recorded (Rettich et al. 1978, Rettich 1982, 2004). Based on our findings, we can confirm that thermophilic *Cx. modestus* became common in wetland areas in southern, eastern, western, and central Bohemia during recent years and it constitutes the major species of mosquito fauna in many places. As a consequence of the recent spread of *Cx. modestus* within the Czech Republic and its willingness to feed on both avian and mammalian hosts, this species appears to be the more appropriate emerging vector of WNV in the Czech Republic.

The greater effectiveness of CO_2-baited CDC traps can be explained by the overall amount of carbon dioxide released from dry ice in comparison to the concentration of CO_2 in the breath of sentinel animals. *Culex pipiens* occurred in both types of traps in relatively similar proportions, whereas *Cx. modestus* was more abundant in sentinel–baited traps. Since both birds and mammals were used as bait, our findings correspond with an assumption of higher mammalophily of *Cx. modestus* and confirm its role as an appropriate WNV bridge vector.

Although several European species of anopheline mosquitoes are important vectors of human malaria (e.g., *Anopheles atroparvus, An. plumbeus, An. sacharovi,* and *An. hyrcanus*), there is virtually no risk of the endemic malaria that was eradicated in the middle of the last century in central Europe, and *Anopheles* surveillance and control programs have been discontinued in many countries. All together, eighteen species of *Anopheles* are recognized in Europe, mainly in the subgenus *Anopheles* (Ramsdale and Snow 2000). *Anopheles* (*Anopheles*) *hyrcanus* (Pallas, 1771), belonging to the *hyrcanus* group, has a wide Palearctic distribution from Spain to China, covering the southern half of Europe, the Mediterranean area, and central Asia. It is typically associated with rice fields that create prolific larval sites, and large populations are frequently associated with rice-growing areas. The Old World *Anopheles hyrcanus* group consists of about 30 known species (Ramsdale and Snow 2000), and some species of this group are important for human health as vectors of malarial parasites and other mosquito-borne diseases in the Oriental and Palearctic regions. The European distribution of the western forms of *An. hyrcanus* includes several southern European countries (Ramsdale and Snow 2000) and was recently described from Slovakia (Halgoš and Benková 2004). New records from southern Moravia represent the northern point of *An. hyrcanus* occurrence in Europe. Our findings correspond with the previous findings of Halgoš and Benková (2004) in Slovakia and clearly demonstrate further spreading of this Mediterranean species involving malaria transmission within the temperate zone of central Europe. During the last three years, *An. hyrcanus* was repeatedly found in relatively high densities in all studied sites in southern Moravia and it is capable of becoming established in new territories.

Our findings support the view that the spread of newly-emerging Mediterranean mosquito species through temperate central Europe could be responsible for vector-borne disease. In southern Moravia, we have identified a new Mediterranean mosquito species, *An. hyrcanus,* and using historical data we demonstrated the substantial spreading of another Mediterranean species, *Culex modestus*, into the Czech Republic.

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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 CO_2-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 CO_2-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 CO_2-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

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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 Ťahyňa 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 (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 (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 (CO_2). 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 CO_2-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 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′-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 Log_{10} 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, Petrusek 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 CO_2 traps was done using Pearsons 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 CO_2-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) ± SE, and proportion of the total catch (%)

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

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

Spatial Distribution. During 2006 and 2007, transects of CO_2 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

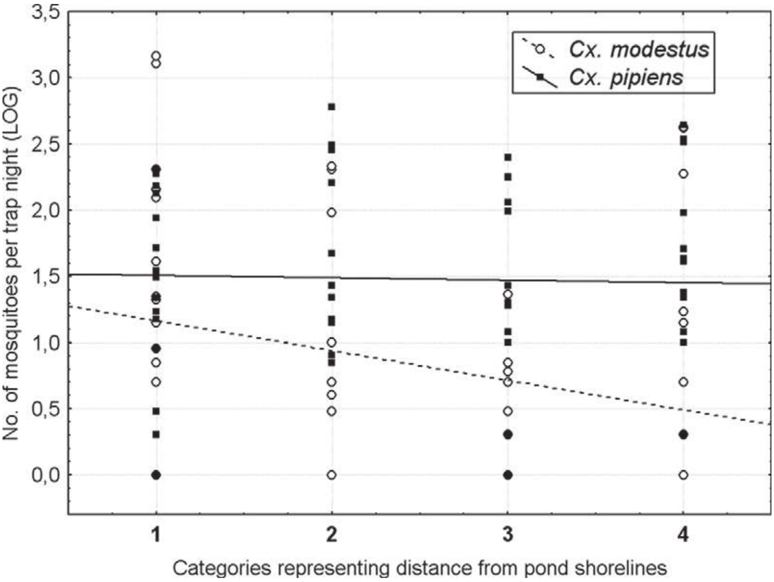

Fig. 1. Scatterplot of numbers of *Cx. pipiens* and *Cx. modestus* females captured per trap night by using transect lines. The traps were divided into four categories according to biotope and distance from water shorelines: 1) reeds (up to 50 m), 2) boundary of reeds with surrounding biotopes, 3) neighboring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

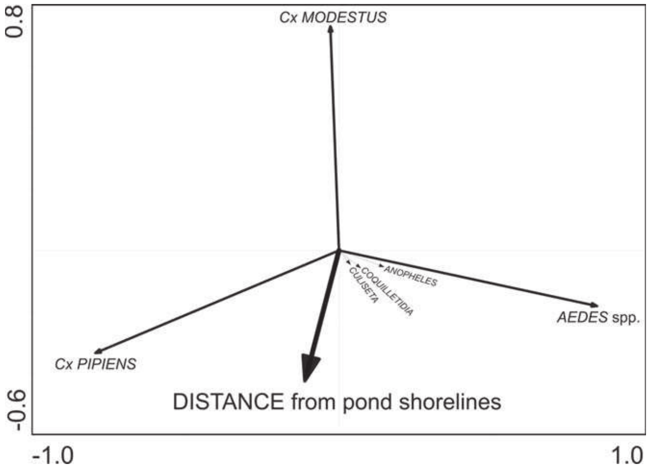

Fig. 2. Relationships between abundance of mosquito species and distance from fishpond shorelines. Results were obtained by partial redundancy analysis of the spatial distribution of mosquito species along the distance from breeding sites using principal component analysis (PCA; CANOCO). Data from 2005 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 *Cx. pipiens*	A	A	A	A	C	A	A	C	T	A	C
99–222 1999 *Cx. pipiens*	·	·	·	·	·	·	·	·	·	·	T
06–222 2006 *Ae. rossicus*	·	·	·	·	·	·	·	·	·	G	·
Re-1–2006 *Cx. modestus*	G	·	G	·	·	·	·	·	·	G	·
Re-2–2006 *Cx. modestus*	G	·	G	·	·	·	·	·	·	G	·
Re-3–2006 *Cx. modestus*	G	·	G	·	·	·	·	·	·	G	·
Re-4–2006 *Cx. modestus*	G	·	G	·	·	·	·	·	·	G	·
Nr-1–2006 *Cx. pipiens*	G	·	G	G	·	·	·	·	A	G	·
Nr-2–2007 *Cx. pipiens*	G	·	G	·	·	·	·	·	·	G	·
Nr-8–2007 *Cx. pipiens*	·	·	·	·	·	·	·	·	·	·	·
Nes-1–2007 *Cx. pipiens*	G	·	G	·	·	·	G	T	·	G	·
Mu-1–2006 *Cx. pipiens*	G	T	G	·	·	G	·	·	·	G	·
Mu-2–2006 *Cx. pipiens*	G	·	G	·	T	·	·	·	·	G	·
Mu-3–2006 *Cx. pipiens*	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 ornithophagic, 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 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 (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 bloodmeal identifications confirmed our results from animal-baited traps, mammalian blood was found in only a small proportion of engorged *Cx. pipiens* females captured by CO_2-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 CO_2-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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Trypanosoma culicavium sp. nov., an avian trypanosome transmitted by *Culex* mosquitoes

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A novel avian trypanosome, *Trypanosoma culicavium* sp. nov., isolated from *Culex* mosquitoes, is described on the basis of naturally and experimentally infected vectors and bird hosts, localization in the vector, morphological characters and molecular data. This study provides the first comprehensive description of a trypanosome species transmitted by mosquitoes, in which parasites form plugs and rosettes on the stomodeal valve. Trypanosomes occurred as long epimastigotes and short trypomastigotes in vectors and culture and as long trypomastigotes in birds. Transmission of parasites to bird hosts was achieved exclusively by ingestion of experimentally infected *Culex* mosquito females by canaries (*Serinus canaria*), but not by Japanese quails (*Coturnix japonica*), nor by the bite of infected vectors, nor by ingestion of parasites from laboratory cultures. Transmission experiments and the identity of isolates from collared flycatchers (*Ficedula albicollis*) and *Culex* mosquitoes suggests that the natural hosts of *T. culicavium* are insectivorous songbirds (Passeriformes). Phylogenetic analyses of small-subunit rRNA and glycosomal glyceraldehyde-3-phosphate dehydrogenase gene sequences demonstrated that *T. culicavium* sp. nov. is more related to *Trypanosoma corvi* than to other avian trypanosomes (e.g. *Trypanosoma avium* and *Trypanosoma bennetti*).

INTRODUCTION

Mosquitoes are among the most important vectors of human and animal pathogens (viruses, bacteria, protozoa and filariae) worldwide. However, relative to other biting flies, mosquitoes have been poorly studied as vectors of trypanosomes (genus *Trypanosoma*; family Trypanosomatidae; order Kinetoplastea), widespread blood parasites of vertebrates. Although several well-known trypanosome species are causative agents of important diseases (e.g. Chagas disease, sleeping sickness and nagana), the majority of extant trypanosome species remains overlooked. Avian trypanosomes, for instance, are a much less well-known group of trypanosomes, even though almost 100 trypanosome species have been described to date (Baker, 1976; Bennett *et al.*, 1982; Podlipaev, 1990; Sehgal *et al.*, 2001). The biology of avian trypanosomes (vectors, life cycles, host and vector specificity) is poorly known or totally unknown, despite the fact that some species could be pathogenic to their host (Molyneux *et al.*, 1983).

Experimental transmission of parasites belonging to the *Trypanosoma avium* clade from insect vectors to bird hosts has been achieved using the blackflies *Eusimulium latipes* (Votýpka & Svobodová, 2004) and *Simulium rugglesi* (Desser *et al.*, 1975), whereas the hippoboscid fly *Ornithomyia avicularia* was shown to transmit parasites of the *Trypanosoma corvi* clade (Baker, 1956; Mungomba *et al.*, 1989). Vectors of avian trypanosomes from the *Trypanosoma bennetti* clade are still unknown. Although mosquitoes have been considered several times as vectors of avian trypanosomes (for the first time in 1904 by Schaudinn, who observed division of epimastigotes in the midgut and hindgut of *Culex pipiens* females blood-fed on an infected owl), and several transmission experiments involving mosquitoes and birds have been performed (David & Nair, 1955; Bennett, 1961; Chatterjee, 1977), the role of mosquitoes in the transmission of avian trypanosomes in nature has not yet been demonstrated unambiguously.

Abbreviations: gGAPDH, glycosomal glyceraldehyde-3-phosphate dehydrogenase; p.i., post-infection; SSU, small subunit.

The GenBank/EMBL/DDBJ accession numbers for the SSU rRNA gene sequences reported in this paper are HQ107966–HQ107970, HQ909083 and HQ909084 for *Trypanosoma culicavium* sp. nov. isolates PAS109, CUL28, CUL31, PAS99, CUL6, CUL24 and CUL30, respectively. Those for the gGAPDH gene sequences reported in this paper are HQ906657–HQ906661 for *T. culicavium* sp. nov. isolates CUL1, PAS99 and PAS109, *T. corvi* OA6 and *T. avium* ANI14A, respectively.

A supplementary table is available with the online version of this paper.

In dixenous trypanosomatids, parasite transmission is accomplished either by the bite of blood-sucking vectors, in which parasites develop in the fore part of the alimentary tract (e.g. leishmanias in phlebotomine sandflies) or in salivary glands (e.g. *Trypanosoma brucei* in tsetse flies), or by the contamination of host skin and/or conjunctiva by vector faeces during and after blood sucking of vectors, where parasites develop in the hind part of the vector's digestive tract (e.g. *Trypanosoma cruzi* in kissing bugs). Much less is known about the transmission of avian trypanosomes, some of which accomplish transmission by ingestion of infected vectors or the contamination of abraded skin or host conjunctiva by parasites present in vector faeces (the hippoboscid fly *Ornithomyia avicularia* and blackfly *Eusimulium securiforme*) (Baker, 1956; Mungomba *et al.*, 1989; Desser *et al.*, 1975; Votýpka & Svobodová, 2004).

Since morphological identification of trypanosomes is impossible (Desquesnes & Dávila, 2002), molecular techniques have been used to identify trypanosomes from a number of animals. According to recent molecular phylogenetic studies (Votýpka *et al.*, 2002, 2004; Hamilton *et al.*, 2007), bird trypanosomes form three distinct clades named after the principal species: *T. avium*, *T. corvi* and *T. bennetti*. Several recent studies have presented data on the occurrence of avian trypanosomes in blackflies, hippoboscid flies and mosquitoes (Votýpka *et al.*, 2002, 2004; Votýpka & Svobodová, 2004; Van Dyken *et al.*, 2006). Species identification of avian trypanosomes and identification of their host range require matching the trypanosomes found in vectors with those in vertebrate hosts. In our previous study (Votýpka *et al.*, 2002), we compared small-subunit (SSU) rRNA gene sequences to confirm ornithophilic simuliids as probable vectors of bird trypanosomes, which was later confirmed experimentally (Votýpka & Svobodová, 2004). Moreover, one mosquito isolate marked as CUL1 belonged to the genus *Trypanosoma*, but was not similar to *T. avium* sensu stricto isolated from blackflies (Votýpka *et al.*, 2002). In this study, we formally describe a novel trypanosome species, *Trypanosoma culicavium* sp. nov., compare it with other avian and mosquito trypanosomes and elucidate its mode of transmission from the insect vector to the bird host.

METHODS

Detection of parasites from mosquitoes and birds. Mosquitoes were collected in the framework of two independent studies. In the first study, in 1998–2002, dissection to determine the presence of trypanosomes and subsequent cultivation was carried out on mosquitoes collected at several localities in the Czech Republic (Prague, southern Moravia and southern Bohemia). In the second study, in 2005–2007, using mosquitoes collected in southern Bohemia and southern Moravia, trypanosomes in mosquitoes were detected by PCR only.

Bird blood samples were taken from adult collared flycatcher (*Ficedula albicollis*) females trapped in nest-boxes during the breeding season in the Milovický forest, southern Moravia. Methods of mosquito collection and dissection, bird blood sampling and parasite isolation used in the present study have been described elsewhere (Votýpka *et al.*, 2002, 2008; Votýpka & Svobodová, 2004).

RAPD and phylogenetic analyses. Total DNA samples were extracted from cultured trypanosome lines using a DNA tissue isolation kit (Roche) according to the manufacturer's instructions. DNA samples of mosquito isolates were evaluated by RAPD analysis as described by Svobodová *et al.* (2007). SSU rRNA gene loci of selected isolates were sequenced by using universal eukaryotic primers Medlin A (5′-CTGGTTGATCCTGCCAG-3′) and Medlin B (5′-TGATCCTTCTGCAGGTTCACCTAC-3′). Glycosomal glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) genes from the isolates were amplified using primers G3 (5′-TTYGCCGYATYGGYCGCATGG-3′) and G4a (5′-GTTYTGCAGSGTCGCCTTGG-3′) as described by Hamilton *et al.* (2004). The PCR products of both genes were purified and sequenced directly. A dataset containing 43 gGAPDH sequences of trypanosomatids was created, including sequences of *T. culicavium*.

Phylogenetic analyses of the two analysed genes (including isolate CUL1, of which the SSU rRNA gene sequence was published previously under GenBank accession no. AF416561) were performed as described by Zídková *et al.* (2010); phylogenetic trees were reconstructed using maximum-parsimony (PAUP* 4.0b10), maximum-likelihood (PhyML win32) and Bayesian (MrBayes 3.1.1) methods. Sequences used for phylogenetic analyses are detailed in Supplementary Table S1, available in IJSEM Online.

PCR screening for trypanosomes in mosquitoes. For PCR screening of trypanosomes, calculation of prevalence and phylogenetic analyses, we used protocols described by Van Dyken *et al.* (2006) and Votýpka *et al.* (2008). Mosquitoes were sorted into species and unfed females were frozen at −70 °C in pools of 10–50 for later processing. Pooled females were homogenized in RNA*later* RNA stabilization reagent (Qiagen) and total DNA was extracted as described above.

Pooled female mosquitoes were tested for the presence of trypanosomes using specific trypanosome PCR primers to amplify 18S rRNA gene. Primers used for amplification were R-221 (5′-GGTTCCTTTCCTGATTTACG-3′) and Medlin B (5′-TGATCCTTCTGCAGGTTCACCTAC-3′). As a negative control, DNA from a laboratory colony of *Culex quinquefasciatus* was used. Amplicons of 1300–1400 bp were gel-isolated and directly sequenced. Homologous 18S rRNA gene sequences originated from our previous studies (Votýpka *et al.*, 2002, 2004) or were downloaded from the NCBI database. Newly obtained sequence data were aligned and compared using CLUSTAL_X version 1.81 to find the most closely related trypanosome 18S rRNA gene sequences.

Experimental infection of mosquitoes and transmission to birds. All animal experiments were performed according to Czech law and Guidelines on Animal Experimentation. Trypanosome isolate CUL1 from mosquito was used for infectious experiments with mosquitoes as well as with birds; bird isolate PAS99 was used only for infection of mosquitoes. In several independent experiments, 10- to 14-day-old adult female mosquitoes of the laboratory reared colony of *C. quinquefasciatus* (a member of the *Culex pipiens* complex) were infected by feeding through a chick skin membrane on heat-inactivated rabbit blood containing a 7- to 14-day-old culture of 10^6–10^7 CUL1 parasites ml^{-1}. In a single experiment, the same procedure was followed with *C. quinquefasciatus* and isolate PAS99 and with *Aedes aegypti* and isolate CUL1 (10^7 parasites ml^{-1} in both experiments). Fed females were separated 1 day after blood feeding. Mosquitoes were maintained in cages at 18–20 °C, 80 % relative humidity and supplemented with 50 % sucrose solution. Mosquito females were dissected 12–28 days post-infection (p.i.) and examined under a light microscope for determination of the location and density of flagellates in the gut.

Three different methods were used for experimental infection of laboratory reared canaries (*Serinus canaria*, Passeriformes) and Japanese quails (*Coturnix japonica*, Galliformes): (i) by bite of experimentally infected mosquitoes, (ii) by ingestion of experimentally infected mosquitoes and (iii) by ingestion of trypanosome culture forms (see Table 1). All experimental birds were negative for trypanosomes before experimental infections.

In method (i), mosquito females, 10–20 days after the first infectious feeding, were allowed to feed again on nine canaries and five Japanese quails. Immediately after this second feeding, fed females were separated and dissected and their guts and stomodeal valves were checked microscopically. In method (ii), guts from infected mosquitoes, 2–3 weeks after infection, were homogenized in saline and were applied perorally to canaries and Japanese quails. In method (iii), canaries and Japanese quails were inoculated perorally with 10^7 trypanosomes from a 7-day-old culture.

The presence of trypanosomes in experimental birds was checked at 15, 40, 60 and 240 days p.i. by *in vitro* cultivation of blood on SNB-9 blood agar and by xenodiagnosis. For Japanese quails, the experiments were terminated at 60 days p.i., and their femur bone marrow was used for cultivation on SNB-9 blood agar. For xenodiagnosis and a transmission cycle investigation, naive mosquito females of *C. quinquefasciatus* were allowed to suck blood on experimentally infected canaries and Japanese quails 3 weeks after experimental infection. Fed females were separated 1 day after blood feeding and were dissected and microscopically inspected 9 days after blood-feeding.

Light and electron microscopy. Light-microscope preparations were made from cultured cells (a 5- to 7-day-old culture in exponential phase) of isolates CUL1, CUL6, CUL30, PAS99 and PAS109 (*T. culicavium*) and ITMAP and OA6 (*T. corvi*), on cells (CUL1) extracted from experimentally infected mosquitoes during their dissection (10 days p.i., when a majority of parasites are attached to the stomodeal valve) and from blood films prepared from birds experimentally infected by the CUL1 strain (30 days p.i.; only two trypanosomes were seen). For parasite measurements, the QuickPHOTO MICRO software (Olympus) and Gr-measure software, CCD camera DP70 and Olympus BX51 microscope were used. Morphometric data of cell length were obtained for 25 cells of each morphotype: cultured cells of all studied isolates and trypanosomes from experimentally infected mosquitoes in the case of CUL1. Measurements (in μm) included cell length and (for CUL1) cell width at the widest point and length of the free flagellum. Different morphological types from culture and experimentally infected mosquitoes were compared using ANOVA post-hoc comparisons (Statistica software). Electron-microscope preparations from 5- to 7-day-old cultures and measurement of the kinetoplast thickness of the mosquito (CUL6) and bird (PAS99) isolates were made as described by Votýpka *et al.* (2002).

RESULTS AND DISCUSSION

Origin and prevalence of *Trypanosoma culicavium* sp. nov. in mosquitoes

Trypanosoma culicavium (isolate ICUL/CZ/1998/CUL1) was isolated from the stomodeal valve of a *Culex pipiens* (Culicidae, Diptera) mosquito female captured in southern Moravia (Milovický forest: 48° 50′ N 16° 42′ E, 290 m above sea level) in 1998. Altogether, 1032 specimens of mosquito females (899 of *C. pipiens* and 133 of *Culex modestus*) captured in southern Moravia, southern Bohemia and the vicinity of Prague city were dissected, and 31 kinetoplastid strains were established. Of those, 23 strains were *T. culicavium* according to RAPD analysis (data not shown). Twenty-two isolates originated from *C. pipiens* (prevalence 2.4 %) and one (CUL30) from *C. modestus* (prevalence 0.7 %); 14 isolates originated from Prague, five from southern Moravia and four from southern Bohemia. In the next step, six of these isolates [one Prague isolate (CUL24), two Moravian isolates (CUL1 and CUL6) and three southern Bohemia isolates (CUL28, CUL30 and CUL31)] were sequenced and shown to be identical to *T. culicavium* by sequence analyses of the SSU rRNA gene (see Fig. 4b; Table 2). Avian trypanosomes are transmitted by diverse vectors; however, the mere presence of trypanosomes in a blood-sucking invertebrate does not necessarily incriminate it as a vector. Only if parasites survive defecation of the rest of a blood-meal and successfully colonize a digestive tract (or salivary glands) can we speculate about transmission to vertebrate hosts. In our case, mature infections were localized to the stomodeal valve, and all naturally infected mosquito females had no blood in their intestine.

In conjunction with trypanosome PCR screening among mosquitoes captured around fishponds in southern Moravia and Bohemia, 129 pools representing 2490 individuals of *Culex* (*Culex*) *pipiens*, 3034 of *Culex* (*Barraudius*) *modestus* and 41 of *Culex* (*Culex*) *torrentium* were tested. Altogether, 11 pools were positive for trypanosome DNAs and, of those, only seven (5.5 % of the total) were *T. culicavium* by sequencing of part of the SSU rRNA gene. It can be supposed that only a single specimen within each positive pool was infected. The prevalence of tested mosquitoes was 0.3 % for *C. pipiens* and 0.05 % for *C. modestus*. All specimens of *C. torrentium* were negative. Since the PCR-tested mosquitoes had no blood in their guts, we suppose that mosquito females had not been simply carrying replicating trypanosomes in an undigested blood-meal.

The presence of parasites found by dissection and by PCR demonstrates that *T. culicavium* occurs in *Culex* mosquitoes (*C. pipiens* and *C. modestus*) at various localities (forest areas and wetlands) in Bohemia and Moravia. Our data, based on

Table 1. Set-up of experimental transmission of trypanosomes to bird hosts

Route of infection	Infection dose	Canaries*	Quails*
(i) By bite	2–14 infectious *Culex*	9/0	5/0
(ii) Perorally	7–15 infectious *Culex*	8/3	4/0
(iii) Perorally	10^7 culture forms	4/0	3/0

*Numbers of experimental/positive birds.

Table 2. Isolates of *T. culicavium* originating from *Culex* mosquitoes and collared flycatchers

Isolate	Host species	Locality	Year	GenBank accession no.	
				SSU rRNA gene	gGAPDH
CUL1	*Culex pipiens*	Southern Moravia	1998	AF416561	HQ906657
CUL6	*Culex pipiens*	Southern Moravia	2000	HQ107970	ND
CUL24	*Culex pipiens*	Prague	2001	HQ909083	ND
CUL28	*Culex pipiens*	Southern Bohemia	2002	HQ107967	ND
CUL30	*Culex modestus*	Southern Bohemia	2002	HQ909084	ND
CUL31	*Culex pipiens*	Southern Bohemia	2006	HQ107968	ND
PAS99	*Ficedula albicollis*	Southern Moravia	2006	HQ107969	HQ906658
PAS109	*Ficedula albicollis*	Southern Moravia	2007	HQ107966	HQ906659

ND, Not determined.

naturally infected mosquitoes, indicate that *T. culicavium* is able to infect at least two different subgenera of the genus *Culex*. The three- to sixfold lower prevalence of *T. culicavium* in *C.* (*Barraudius*) *modestus* could indicate that mosquitoes from the subgenus *Culex* (e.g. *C. pipiens*) are more susceptible hosts for *T. culicavium* than mosquitoes of other subgenera, or that these species feed on different hosts (J. Votýpka, unpublished results).

Molecular detection of avian trypanosomes (belonging to the *T. avium* and *T. corvi* groups) in wild-caught vectors was reported by Van Dyken *et al.* (2006), who found three (0.2 %) positive *Culex pipiens* and *Culex* (*Culex*) *tarsalis* mosquitoes.

Experimental infections and transmissions

For several infection experiments with isolate CUL1, more than 1000 mosquito females from a laboratory reared colony of *C. quinquefasciatus* were used. The infection rate after blood feeding through a chicken-skin membrane was 60–85 %. Trypanosomes were localized mainly on the stomodeal valve, and the majority of positive mosquito females were heavily infected (i.e. more than 1000 parasites per gut). Thus, *C. quinquefasciatus* was shown to be a good experimental host of *T. culicavium*. To prove transmissibility of avian isolates of *T. culicavium*, 70 females of *C. quinquefasciatus* were fed with bird isolate PAS99. After 14–28 days, 26 mosquitoes (37 %) were found to be infected with parasites attached to the stomodeal valve.

To assess the vector specificity of *T. culicavium*, 92 females of *A. aegypti* were fed with CUL1 isolate. Only two specimens (2 %) were found to be positive with parasites attached to the stomodeal valve, one of them being heavily infected. Control *C. quinquefasciatus* females fed on the same inoculum of trypanosomes were infected in 66 % of cases. This demonstrates that genera other than *Culex* (the natural hosts) are able to become infected under experimental conditions, albeit with a very low infection rate. Information about vector specificity of avian trypanosomes is very limited and inconsistent (see David & Nair, 1955; Baker, 1956; Bennett, 1961, 1970; Chatterjee, 1977; Miltgen & Landau, 1982; Van Dyken *et al.*, 2006; Reeves *et al.*, 2007). We suppose that *T. culicavium* is not identical to a trypanosome from *Aedes* mosquitoes described by David & Nair (1955), which occurred in the hindgut. The ability of *T. culicavium* to develop in the intestine of *Culex* mosquitoes from two subgenera (*Culex* and *Barraudius*) indicates a host range that extends over different mosquito subgenera, but probably not genera.

The development of avian trypanosomes has been demonstrated in several dipteran species. In blackflies, epimastigotes replicate during blood-meal digestion in the midgut and, after peritrophic membrane rupture, the epimastigotes migrate to the hindgut and rectum, where they attach to a chitinous intima via hemidesmosomes (Bennett, 1970; Desser, 1977; Dirie *et al.*, 1990; Votýpka & Svobodová, 2004). The transmission of parasites to a new host can occur via vector ingestion or defecation, and possibly via urination (diuresis) (Dirie *et al.*, 1990; Votýpka & Svobodová, 2004). Transmission by vector ingestion was also demonstrated for hippoboscid flies by Baker (1956) and hemidesmosome-like adhesion for trypanosomes in the posterior part of the alimentary tract of *Ornithomyia avicularia* was reported by Mungomba *et al.* (1989).

T. culicavium (isolate CUL1) was shown to occur first in the mosquito midgut; later, the parasite moves to the anterior part of the gut, attaches to the stomodeal valve (Fig. 1) and degrades it in a way similar to leishmania parasites in sandfly vectors (Volf *et al.*, 2004). It has been speculated that the mode of transmission to birds is probably regurgitative, the ingested blood and trypanosomes being regurgitated, as a result of the less functional proventriculus, back into the bite wound during feeding (Volf *et al.*, 2004).

Our experiments, focused on the transmission mode of trypanosomes from insect vectors to bird hosts, resulted in successful transmission only in the case of experimentally infected *Culex* mosquitoes ingested by canaries; all birds inoculated with trypanosome stages from culture or bitten by infected mosquitoes were negative (Table 1).

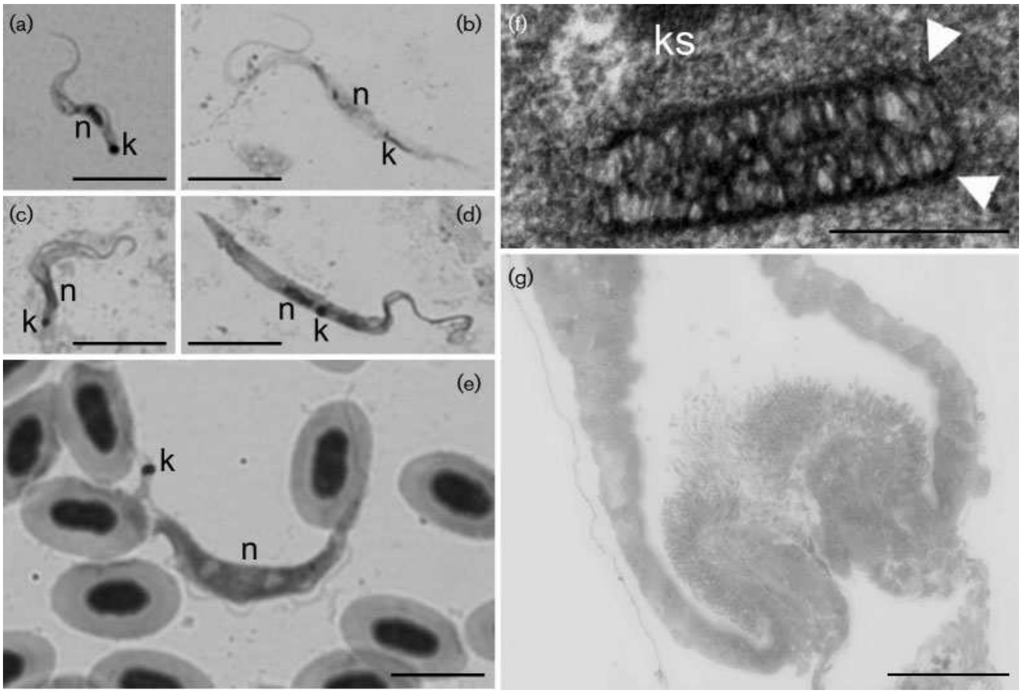

Fig. 1. (a–e) Micrographs of Giemsa-stained *Trypanosoma culicavium* showing short trypomastigote (a, c) and long epimastigote (b, d) forms from the stomodeal valve of experimentally infected *Culex* mosquito females 10 days after infection (a, b) and from a 7-day-old culture (c, d) and long trypomastigote from the blood of an experimentally infected canary at 30 days p.i. (e). (f) Transmission electron micrograph of kinetoplast with the characteristic low-pitched and elongated shape. k, Kinetoplast; ks, kinetosome of the flagellum; n, nucleus. Arrowheads indicate the thickness of the kinetoplast. (g) Semi-thin section of the stomodeal valve with attached parasites in experimentally infected *Culex* mosquito females, 10 days p.i. Bars, 10 μm (a–e), 0.5 μm (f) and 50 μm (g).

Trypanosomes were found by xenodiagnosis in the blood of three of eight canaries that were infected perorally with gut forms of parasites from experimentally infected mosquito females. In these three positive canaries, the infection rates of the xenodiagnosis were 82 % (23 of 28 specimens; 45 days p.i.), 10 % (1 of 10; 17 days p.i.) and 8 % (1 of 12; 22 days p.i.). The mosquito females were dissected after defecation to prove that the infection persisted after complete digestion of the blood-meal, and parasites were found in their stomodeal valves in all cases. The presence of trypanosomes was confirmed by *in vitro* cultivations only in one xenodiagnosis-positive canary (with the highest mosquito infection rate, of 82 %), and parasites were detected from day 15 to day 40 p.i. Later cultivations were negative, probably because of the disappearance of trypanosomes from peripheral blood.

The changes of the mosquito stomodeal valve resemble those seen in sandflies, where they are suggested to facilitate transmission when biting (Volf *et al.*, 2004). Therefore, we originally supposed that *T. culicavium* was transmitted by mosquito bite. In order to find out which morphological forms of *T. culicavium* are regurgitated, infected mosquitoes were force-fed using a Hertig apparatus (Hertig & McConnell, 1963); however, no trypanosomes were regurgitated. Moreover, after feeding infected mosquitoes on a chick-skin membrane, no trypanosomes were found in the blood used for feeding. Other indirect evidence came from our field studies. Buzzards were readily attacked by *Culex* mosquitoes and, although the prevalence of trypanosomes in mosquitoes was as high as 10 %, *T. culicavium* was never isolated from the blood of raptors (J. Votýpka and others, unpublished results; Černý *et al.*, 2011). These results, together with transmission experiments to canaries as well as the isolation of *T. culicavium* exclusively from mosquito-eating passerines, suggests that inoculation by bite does not play a significant role in natural transmission and that *T. culicavium* is indeed transmitted in the first instance by ingestion of infected mosquitoes.

Our experiments proved unambiguously that *T. culicavium* (CUL1 isolate) is transmissible to *Culex* mosquitoes by feeding on an infected bird and demonstrated the successful completion of the *T. culicavium* life cycle. In addition, similar infection rates of *Culex* mosquitoes infected by either artificial feeding through a chicken skin membrane (70–85 %) or by naturally feeding on an infected bird host (up to 82 %) demonstrate that the prevalence in experimentally infected mosquitoes mimics natural infection.

Isolation of *T. culicavium* from wild bird hosts

Our results based on experimental infections and transmission mode suggested the role of insectivorous songbirds in the life cycle of *T. culicavium*. Collared flycatchers (*Ficedula albicollis*) are reported to feed on mosquitoes (Hudec & Šťastný, 2005), and 21 of 56 sampled birds were positive for *Trypanosoma* sp. Twelve isolates were established; of these, two isolates (PAS99 and PAS109) were identical to *T. culicavium* (isolate CUL1) as shown by sequencing of the SSU rRNA gene and gGAPDH (see Fig. 4; Table 2).

The transmission of *T. culicavium* under laboratory conditions occurs by ingestion and this, together with the fact that flycatcher isolates (PAS99 and PAS109) are identical to CUL1 by sequencing, suggests that the natural hosts of *T. culicavium* are insectivorous songbirds (Passeriformes).

Light microscopy and transmission electron microscopy

Two morphologically different types of trypanosome (isolate CUL1) were present on slides obtained from mosquito stomodeal valves as well as on slides from *in vitro* cell cultures (isolates CUL1, CUL6, CUL30, PAS99 and PAS109). Trypomastigotes were the shorter observed morphotypes, whereas the longer morphotypes were epimastigotes (Fig. 1). For isolate CUL1, body lengths of short (12.0 ± 0.6 μm, $n=25$) as well as long (24.3 ± 2.6 μm, $n=25$) insect forms were significantly shorter (ANOVA; $F_{(1,\ 50)}=29.81$; $P<0.001$ and $F_{(1,\ 50)}=36.66$; $P<0.001$) than culture forms of trypomastigotes (13.5 ± 0.7 μm, $n=25$) and epimastigotes (30.8 ± 3.7 μm, $n=25$), respectively (Fig. 2). Significant differences were not found among lengths of cultured cells of five isolates of *T. culicavium* (CUL1, CUL6, CUL30, PAS99 and PAS109), nor for the short trypomastigote form (ANOVA; $F_{(4,\ 125)}=0.19$; $P=0.94$) nor for the long epimastigote form (ANOVA; $F_{(4,\ 125)}=1.09$; $P=0.36$).

Baker (1966) observed similar morphotypes ('big epimastigotes and small trypomastigotes') in a culture of trypanosomes obtained from a rook. In contrast, we observed only one morphotype (epimastigotes) in a culture of *T. corvi* (isolate OA6 and LSHTM/ITMAP 180795), and the cell length was significantly different (ANOVA; $F_{(2,\ 300)}=450.72$; $P<0{,}001$) from that of *T. culicavium* (shorter than epimastigotes and longer than trypomastigotes; Fig. 3). It is possible that Baker worked initially with *T. culicavium* (which exhibited two morphotypes); nevertheless, the strain originated from a rook and deposited as LSHTM belongs to *T. corvi*, with one morphotype only.

Only two trypomastigotes were found on a Giemsa-stained thin blood smear of experimentally infected canary (Fig. 1). The utility of delineating trypanosome species based on morphometrics of blood-stream forms is, however, questionable, as pleomorphism of numerous trypanosomes has been documented (Baker, 1976).

Culture forms of *T. culicavium* were also examined by transmission electron microscopy (Fig. 1). We have shown previously (Lukeš & Votýpka, 2000) that the kinetoplast thickness and the minicircle size vary significantly among avian trypanosomes (e.g. *T. corvi*, 0.37 μm; *T. avium*, 0.69–0.85 μm). The kinetoplast thickness of *T. culicavium* mosquito isolate CUL1 [0.310 ± 0.031 μm; $n=35$; see our previous study (Votýpka *et al.*, 2002)] was similar to that of newly measured bird (PAS99) and mosquito (CUL6) isolates [respectively 0.331 ± 0.045 μm ($n=38$) and 0.299 ± 0.023 μm ($n=40$)], confirming the identity of the isolates.

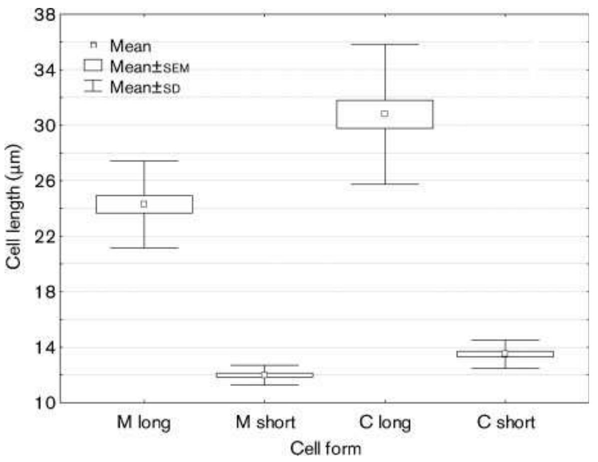

Fig. 2. Comparison of cell lengths of long and short forms of *T. culicavium* isolate CUL1 originating from experimentally infected *C. quinquefasciatus* mosquito females (M) and from culture (C). Twenty-five cells of each type were measured.

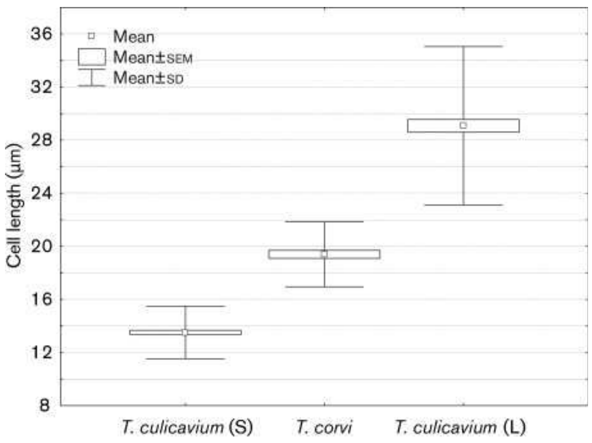

Fig. 3. Comparison of cell lengths of long (L) and short (S) forms of *T. culicavium* isolates CUL1, CUL6, PAS99 and PAS109 and *T. corvi* isolates OA6 and LSHTM. Twenty-five cells of each type and isolate were measured.

Phylogenetic position and comparison with other described species

As shown previously (Votýpka *et al.*, 2002, 2004), SSU rRNA gene nucleotide sequence analysis indicated that the newly described species of the genus *Trypanosoma* (represented by isolate CUL1) clusters with trypanosomes isolated from birds [isolate LSHTM/ITMAP 180795 from rook (*Corvus frugilegus*) caught in the UK] and from a hippoboscid fly [isolate OA6 (*Ornithomyia avicularia*) captured in the Czech Republic], which together form a highly supported *T. corvi* clade. A new phylogenetic tree (Fig. 4) was created on the basis of gGAPDH and SSU rRNA gene sequences, containing all relevant GenBank sequences and sequences of our isolates of *T. culicavium* obtained from mosquitoes (*Culex pipiens*; CUL1 and CUL6 from southern Moravia, CUL28, CUL30 and CUL31 from southern Bohemia and CUL24 from Prague) and two isolates from collared flycatchers (*Ficedula albicollis*; PAS109 and PAS99) captured in southern Moravia. Both groups of isolates, from mosquitoes as well as from birds, formed a highly supported and separate clade together with reference isolate CUL1, closely related to *T. corvi*. The relationship between *T. culicavium* and the *T. corvi* clade was shown unambiguously by a phylogenetic tree based on the SSU rRNA gene sequence (Fig. 4b).

So far, only two species of trypanosomes have been described to occur in mosquitoes. The first is *Trypanosoma rotatorium* (Mayer, 1843), which parasitizes several frog species and is transmitted by *Aedes* or *Culex* mosquitoes. This species is well known and, together with other trypanosomes isolated from amphibians and fish, belongs to a well-supported aquatic clade (Votýpka *et al.*, 2002, 2004; Hamilton *et al.*, 2007). The second species is the avian trypanosome *Trypanosoma noctuae* (Schaudinn, 1904) described from the little owl (*Athene noctua*) in Europe. *C. pipiens* was mentioned in the original description as a vector. Notably, there are several uncertainties in the original description, and Schaudinn (1904) erroneously supposed that the parasite has a sexual phase in the mosquito ovary, assuming that the parasite is congenitally transmitted among mosquitoes. To exclude any possibility of transovarial or transstadial transmission among mosquitoes (without a vertebrate host), several additional laboratory experiments were carried out. Of 197 dissected adult *C. quinquefasciatus* mosquitoes (166 females and 31 males) hatched from eggs oviposited by 12 infected females and reared in water containing dead infected females, we did not record any positive cases.

According to Schaudinn's account, he was dealing with two separate parasites of the little owl, *T. noctuae* and *Trypanosoma ziemanni* (later synonymized with *Leucocytozoon danilewskyi*). The genus *Trypanomorpha* (as well as family Trypanomorphidae) was established by Woodcock (1906) and the only known species so far is the type species, *Trypanomorpha noctuae*; however, the family and genus are not generally accepted nowadays. It is impossible to compare our results with the records of Schaudinn (1904), due to the absence of any relevant description. It has been suggested that the parasite described by Schaudinn from mosquitoes represented monoxenous trypanosomatids of the genus *Crithidia* (Novy *et al.* 1907), and that the parasites isolated by Novy *et al.* (1907) from *Culex* mosquitoes and originally described as '*Trypanosoma* (*Herpetomonas*) *culicis*, n. sp'. represented monoxenous trypanosomatids of the genus *Blastocrithidia* (Wallace & Johnson, 1961).

We therefore describe here a new species of the genus *Trypanosoma*. *Trypanosoma culicavium* sp. nov. is the first named avian trypanosome species to be described from mosquitoes, and the species characters are based on a combination of vector specificity, morphology and, particularly, molecular phylogeny. We suppose that the avian trypanosomes *T. corvi* (isolated from rooks and strictly ornithophilic hippoboscid flies) and *T. culicavium* (from flycatchers and mosquitoes) are different but closely related species. Both the transmission of *T. culicavium* to canaries by ingestion of infected *Culex* mosquitoes and the similarity of trypanosomes obtained from blood of collared flycatchers (*F. albicollis*) to isolates from *Culex* mosquitoes suggest insectivorous songbirds as the natural vertebrate hosts of *T. culicavium*.

Taxonomic summary (based on isolate CUL1)

Diagnosis. Two different morphotypes, short trypomastigotes and long epimastigotes, were observed in the mosquito host as well as in cell culture. Insect forms of experimentally infected *Culex* mosquitoes were 12.0 ± 0.6 μm (n=25) and 24.3 ± 2.6 μm (n=25) long and 1.2 ± 0.2 μm (n=25) and 1.7 ± 0.3 μm (n=25) wide with flagella that were 5.6 ± 0.6 μm (n=25) and 9.3 ± 1.1 μm (n=25) long, respectively. The nucleus was situated in the middle of the cell with the kinetoplast next to it. The thickness of the kinetoplast was 0.310 ± 0.031 μm (n=35).

Trypomastigotes in the blood of experimentally infected canaries were narrow, slightly curved, with a tapering and pointed posterior end. A narrow undulating membrane was located on the external side of the curvature. The length of the body was 40.7–40.9 μm, the width was 3.7–4.1 μm and a free, relatively short flagellum varied in length from 6.8 to 8.0 μm.

Type host (vector). Female of mosquito *Culex pipiens* (Linnaeus, 1758).

Additional host (vector). Female of mosquito *Culex modestus* (Ficalbi, 1890).

Experimental invertebrate host (vector). Female of mosquito *Culex quinquefasciatus* (Say, 1823).

Localization in type host (vector). Stomodeal valve (mature infection).

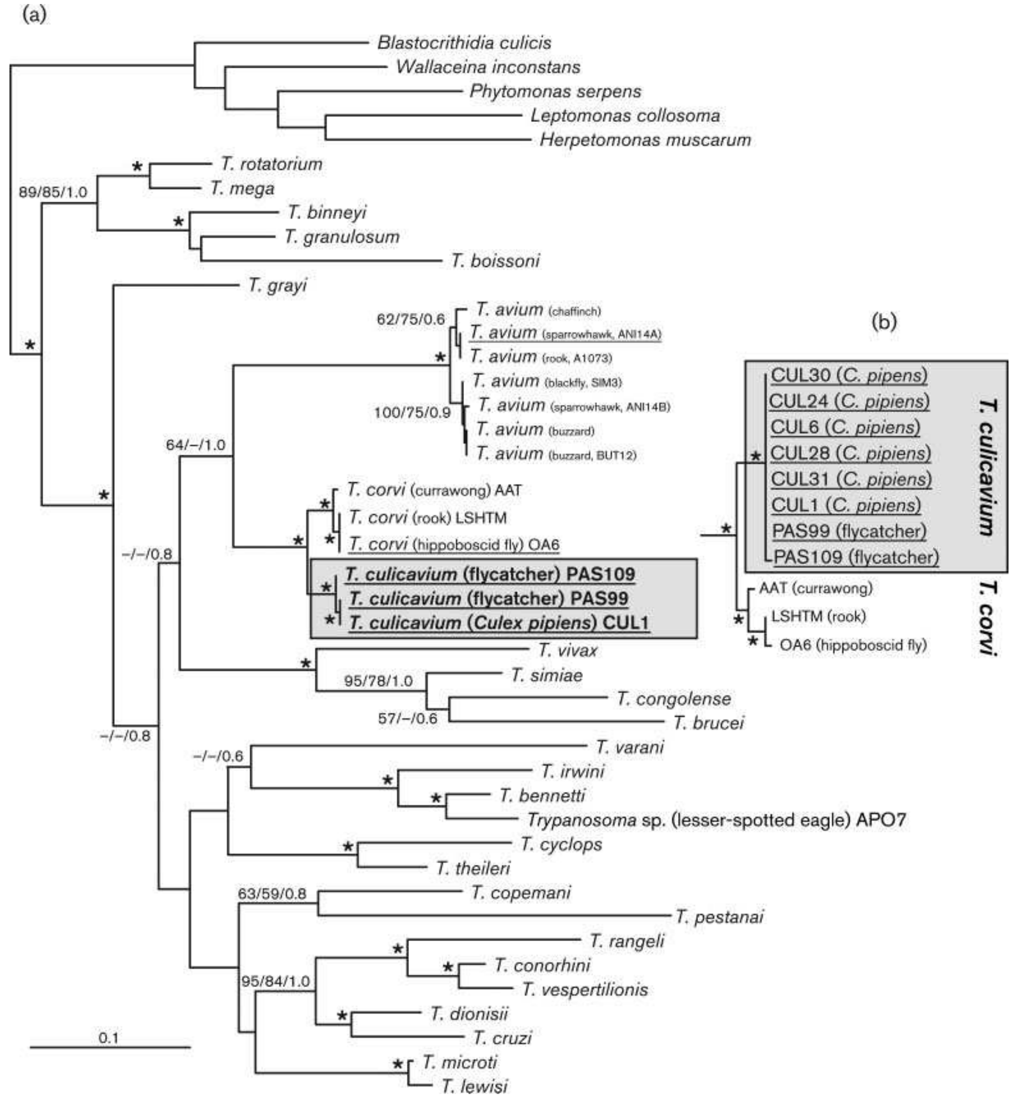

Fig. 4. (a) Maximum-likelihood phylogenetic tree inferred from partial gGAPDH gene sequences including 43 taxa (GenBank accession numbers are indicated). The figure shows topologies obtained by maximum-likelihood using the GTR model for nucleotide substitutions with Γ distribution in 8+1 categories as implemented in PhyML; all parameters were estimated from the dataset. Bootstrap values from maximum-likelihood (1000 replicates) and maximum-parsimony (1000 replicates) and Bayesian support values (posterior probabilities; 5×10^6 generations) are shown at nodes (maximum-likelihood/maximum-parsimony/Bayesian); dashes (–) indicate bootstrap values below 50 % or posterior probability below 0.5 or nodes with different topology. Asterisks (*) at nodes denote Bayesian posterior probabilities and bootstrap percentages of 95 % or higher. Bar, 0.1 substitutions per site. (b) Part of the maximum-likelihood phylogenetic tree inferred from SSU rRNA gene sequences, including 55 taxa (including CUL1). New SSU rRNA gene sequences of *T. culicavium* were obtained for five mosquito isolates (*Culex pipiens*: CUL6, CUL24, CUL28 and CUL31; *C. modestus*: CUL30) and two bird isolates (collared flycatchers, *Ficedula albicollis*; PAS99 and PAS109). The trees were rooted using monoxenous trypanosomatid sequences as an outgroup. Sequences obtained in this study are underlined; accession numbers and strain and source details (where known) are given in Supplementary Table S1.

Presumable natural vertebrate host. Insectivorous songbirds (Passeriformes), e.g. collared flycatcher *Ficedula albicollis* (Temminck, 1815).

Experimental vertebrate host. Canary *Serinus canaria* (Linnaeus, 1758).

Type locality. Southern Moravia (Milovický forest game preserve, 48° 50′ N 16° 42′ E, 290 m above sea level).

Type isolate. CUL1 (ICUL/CZ/1998/CUL1), deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University in Prague, Prague, Czech

Republic, and in the collection of the Academy of Sciences, Biology Centre, Institute of Parasitology, České Budějovice, Czech Republic.

Syntype slides. Giemsa-stained slides of isolate CUL1, both insect and culture forms, deposited in the collection of the Department of Parasitology, Faculty of Science, Charles University in Prague, Prague, Czech Republic.

Etymology. The epithet *culicavium* (cu.li.ca′vi.um) is derived from a name of the insect (vector) and vertebrate hosts. L. n. *culex -icis* a gnat, midge and also a scientific genus name (*Culex*); L. gen. pl. n. *avium* of birds; N.L. gen. pl. n. *culicavium* of *Culex* of birds.

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Seasonal dynamics, parity rate and composition of *Culicoides* attacking game and domestic ruminants

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Abstract

In the light of the emergence of bluetongue disease in Europe, an extensive entomological survey was undertaken to investigate *Culicoides* biting midges (Diptera: Ceratopogonidae) attacking domestic livestock and ruminating hoofed game in the Czech Republic. Within the framework of a bluetongue control program, an intensive and countrywide surveillance program based on the identification of biting midges collected in close proximity to large ungulates was undertaken from 2008 to 2013. Insect sampling using CDC black-light suction traps placed overnight near domestic livestock and hoofed game provides the first comparison of *Culicoides* fauna attacking these two groups of hosts inhabiting different environments. From almost a half million biting midge specimens collected at 41 sampling sites, 34 species were identified including three species newly recorded for the Czech Republic: *Culicoides odiatus, Culicoides clastrieri*, and *Culicoides saevus*. The *Culicoides obsoletus* species complex, incriminated as a bluetongue virus vector, was predominant in both domestic livestock (91%) and hoofed game (52%). A relatively high proportion (around 30%) of parous *C. obsoletus* Meigen females was observed from spring till autumn. In contrast, biting midges were found to be largely absent during at least three winter months, which indicates that the virus overwinters by an alternative mechanism, e.g. in vertebrate hosts or in vector larval stages.

Keywords: *Culicoides*, livestock, hoofed game, dynamics, parity rates

Introduction

Biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) are distributed worldwide, and include more than 1300 described species classified in thirty subgenera and almost forty species complexes (Beckenbach and Borkent 2003). Haematophagous females attack a broad range of mammals including humans and livestock, birds, reptiles and amphibians. Biting midges transmit many parasites including viruses, bacteria, protists and filarial worms. Some play an important role as the vectors of pathogens responsible for diseases with veterinary and public health significance, especially two notable diseases of ruminants and equines, the bluetongue virus (BTV) and African horse sickness (Linley 1985, Mellor et al. 2000, Carpenter et al. 2013).

BTV, the virus that is the focus of this study, has an African origin, and before 1998 bluetongue disease was considered tropical. However, the spread of the Afrotropical vector, *Culicoides imicola* Kieffer, over the Mediterranean basin and the introduction of exotic BT serotypes in northern Europe transmitted secondarily by autochthonous Palaearctic *Culicoides* species, have plunged Europe into a major sanitary crisis (Mellor and Baylis 2009, Velthuis et al. 2010). The first case of BTV emerged in the Czech Republic in 2007 and within the last three years 14 cases of bluetongue disease have been reported (www.svscr.cz). Nevertheless, as of April 2013, the Czech Republic is considered as free of bluetongue disease. Similarly, Schmallenberg virus, a novel *Culicoides*-transmitted ruminant pathogen, has emerged and spread across Europe since 2011 (Balenghien et al. 2014). Since 2012 there have also been cases of Schmallenberg virus confirmed on the Czech territory (www.svscr.cz). Virtually nothing is known about the presence of bluetongue or Schmallenberg virus infections in wildlife in the Czech Republic, but both viruses have been detected in wild ruminants in Europe (García et al. 2008, Linden et al. 2010, 2012).

Culicoides species of veterinary importance usually feed on livestock and horses, and breed in associated habitats. One confirmed (*C. obsoletus*) and several potential (e.g. *Culicoides pulicaris* L, *Culicoides punctatus* Meigen, and *C. nubeculosus* Meigen) vectors of BTV and SBV (Caracappa et al. 2003, Mehlhorn et al. 2007, Saegerman et al. 2008) are present on the territory of the Czech Republic. However, most entomological studies on biting midges in this country are relatively non-recent and did not specifically target species in the proximity of the wild and domestic animals, whose health could potentially be impacted. The biting midge fauna was well described by Orzágh (1980) who enumerated 63 *Culicoides* species for the area of the former Czechoslovakia. After this study, several papers appeared on diversity, biology and abundance of biting midges or describing new species for the Czech Republic (Országh and Chalupský 1987, Knoz and Vaňhara 1991, Knoz 1997, 1998, Knoz et al. 2004, Tóthová et al. 2004, 2005, Knoz and Tóthová 2008); however the majority of these studies focused only on limited parts of the country. The last and the most up-to-date checklist of *Culicoides* species reported 49 species for the Czech lands of Bohemia and Moravia (Tóthová and Knoz 2009).

The present study is based on a countrywide long-term sampling aimed to describe the biting midge fauna, seasonal dynamics, and the occurrence of parous and nulliparous females of the dominant species present in the proximity of livestock (bovids) and ruminating hoofed game (red and fallow deer, mouflon etc.), which may be potentially involved in the life cycle and transmission of BTV and SBV in the Czech Republic.

Materials and methods

Sampling sites and catching procedures

The survey to collect biting midges was conducted at 34 sampling sites with livestock located throughout the Czech Republic (monitored from 2008 to 2013; usually from April to the beginning of December), and eight localities with bred ruminating hoofed game (monitored in 2010 and 2011; from June to August) (Table 1, 2, Fig. 1). Biting midges were collected in the immediate surroundings of cattle farms and in hoofed game farms and preserves or feed-troughs regularly visited by red and fallow deer, mouflon etc. For sampling, CDC black-light suction traps (New Standard Miniature Black-light (UV) Trap, model 1212, John W. Hock Company, Gainsville, Florida) adapted for collecting biting midge, and powered by a 6-V gel battery, were used. In this setup, a suction fan transfers attracted insects to a collection jar with approximately 50 ml of 50-70% ethanol. Traps equipped with photosensors operated for two consecutive nights every two weeks during the sampling seasons. For five selected sampling sites we operated traps throughout the whole year. All traps were affixed outside sheds (for livestock) or near feed troughs (hoofed game), with the lower part of the trap between 1 to 1.5 m above the ground level.

Culicoides identification

After transferring to the laboratory, biting midges were kept in 70% alcohol until subsequent analyses. All specimens were identified to species level based on morphological identification with a stereomicroscope. Doubtful species and species without a wing pattern were mounted into CMCP-9 medium (Polysciences, Warrington, PA) and identified under a light microscope. Several dichotomous keys or species descriptions were used to identify the collected specimens (Országh 1980, Delecolle 1985, www.culicoides.net). Species of the *Culicoides obsoletus* complex were classified using multiplex PCR (Nolan et al. 2007). The identification of species with unspotted wings and species detected for the first time in the Czech Republic were confirmed using barcoding analysis with LCO 1490 and HCO 2198 primers (Folmer et al. 1994) and compared with the BOLD database. To determine the parity rate of *Culicoides obsoletus*, all females obtained from hoofed game collections and from the five selected livestock collecting sites were sorted according their physiological stage using the method of abdomen pigmentation (Dyce 1969).

Data analysis

Original counts (number of individuals) of biting midges were normalized using a Log10 transformation, the normality of data distribution was tested using the Kolmogorov–Smirnov test and analyzed by using chi-square, analysis of variance and regression models (STATISTICA 6.0, StatSoft, Inc., Tulsa, OK), using collection sites and year as main effects.

Results

Culicoides species composition

During our six year survey, CDC traps were run for 3,453 trap/nights during sampling seasons from 2008 to 2013 in cattle farms, and 147 trap/nights in 2010 and 2011 close to semi-wild ungulates in hoofed game preserves. Altogether, 43.2 % (1551) trap/nights contained no *Culicoides*; in contrast, 2.9% (104) traps captured more than one thousand specimens per night. In total, 466.849 *Culicoides* females were collected. The number of *Culicoides* males in our samples was negligible, lower than 0.2 %. It should be noted that *Culicoides* specimens made up a significant proportion of all insects captured by the CDC traps. In both livestock and hoofed game samples, biting midges composed on average 36.3% of all captured insects (from zero up to 100% of the samples; mean: 14.4%; median: 3.3%), and from 1% up to 60%, 8.3% of the total volume of insects (mean: 11.5%; median: 7.3%), respectively.

Morphological identification based on wing patterns evaluated by a stereomicroscope and/or based on the microscopic examination of other characters (e.g. spermathecae, sensilla coeloconica, abundance of mactrotrichie etc.) and supplemented in selected cases by barcoding analysis revealed the presence of 34 species belonging to six subgenera: *Avaritia, Beltranmyia, Culicoides, Monoculicoides, Oecacta*, and *Pontoculicoides* (Table 3). Three new species for the Czech Republic were found during our survey: *Culicoides* (*Oecacta*) *clastrieri* Callot, Kremer & Deduit 1962, *Culicoides* (*Oecacta*) *odiatus* Austen 1921, and *Culicoides* (*Pontoculicoides*) *saevus* Kieffer 1922.

The two most widespread species found in our survey were *Culicoides obsoletus* complex and *Culicoides pulicaris*, recorded in all the sites investigated, followed by *C. puncatus*, absent at just one locality, and *C. pallidicornis* Kieffer, absent at three localities. Occurrences of *Culicoides* species at the studied localities are summarized in the Table 4.

The species *C. obsoletus* s.s. is one of the most common species in Central and Northern Europe, and together with *C. scoticus* Downes & Kettle*, C. dewulfi* Goetghebuer and *C. chiopterus* Meigen forms the *C. obsoletus* complex (Goffredo and Meiswinkel 2004, Savini et al. 2005). Females of this species complex are very similar in morphology, which makes their morphological differentiation almost impossible; however, species identification is possible using multiplex PCR (Nolan et al. 2007). As thousands of *C. obsoletus* complex specimens were collected, and this technique is costly and time-consuming, just a few females from each locality were tested. Muliplex PCR confirmed the presence of three species of this complex, *C. obsoletus* s.s., *C. dewulfi*, and *C. chiopterus* in the Czech Republic. For further analysis, however, the *C. obsoletus* complex was treated as one species.

Comparison of domesticated cattle and hoofed game

Sampling sites in the proximity of cattle farms and hoofed game preserves resulted in 449.093 (mean: 130 per trap; median: 2 per trap) and 17.756 (mean: 120 per trap; median: 23 per trap) *Culicoides* females, respectively. The numbers of biting midges captured per trap (traps with less than ten *Culicoides* specimens captured were excluded from the analysis) varied significantly (p value = 0.013) among years (Fig. 4) for both cattle (mean: 2008 – 332, 2009 – 73, 2010 – 98, and 2011 – 117) and hoofed game (mean: 2010 – 216 and 2011 – 54). The proportion of the most abundant species, *C. obsoletus*, did not significantly differ among years for catches near cattle (2008 – 89.5%, 2009 – 92.6%, 2010 – 90.7%, and 2011 – 92.8%), on the other hand, difference was found in the proportions of the two main *Culicoides* species captured in close proximity to hoofed game: *C. obsoletus* 49.2% in 2010 vs. 69.5% in 2011 and *C. pallidicornis* 21.1% in 2010 vs. 11.5% in 2011.

Significant differences were also found in species compositions. *Culicoides* species and numbers of collected specimens at both types of localities (livestock vs. hoofed game) are presented in Table 3 and Fig. 2. All 34 *Culicoides* species were present in livestock farms, while in the proximity of hoofed game only 20 species where recorded; however it must be taken into account that approximately twenty times fewer biting midges were captured near hoofed game. The most marked differences were found in the proportions of the most abundant species. In both hosts, livestock and hoofed game, the dominant species was *C. obsoletus* with 91.1% and 52.3%, respectively. In livestock, only two other species reached more than one percent: *C. pulicaris* (3.3%) and *C. pallidicornis* (1.0%), while in localities with hoofed game eight species crossed this limit: *C. pallidicornis* (18.8%), *C. pulicaris* (7.4%), *C. nubeculosus* (5.0%), *C. punctatus* (4.0%), *C. reconditus* Campbell & Pelham-Clinton (2.9%), *C. furcillatus* Callot, Kremer et Paradis (2.8%), *C. festivipennis* Kieffer (2.1%), and *C. circumscriptus* Kieffer (1.4%) (Fig. 1, Tab 3).

In addition to the host species targeted (livestock vs. hoofed game), sampling sites also differed in altitude. The highest species richness was found at localities no. 11 (Zásmuky; 307 m a.s.l.) and 18 (Hradištko; 286 m a.s.l.) (Table 4). The most species-poor site was locality no. J7B, KRNAP (The Krkonoše Mountains National Park) with elevation around 1000 m; however because of the outstanding elevation this site was excluded from the analysis. Our overall analysis, however, did not show any significant differences in the number of *Culicoides* species (p = 0.74) or number of captured biting midges (p = 0.45) depending on altitude (Fig. 3 A, B).

Seasonal dynamics of *Culicoides obsoletus*

The seasonal abundance of the most abundant species, *Culicoides obsoletus*, collected at five livestock farms (Nos. 2, 11, 18, 20, and 23) in years 2008 to 2011 is presented in Fig. 4. Numbers of *C. obsoletus* were highest during the spring, but several peaks appeared during other seasons in all four studied years. In all analyzed years, numbers of *C. obsoletus* significantly decreased in late October and the latest specimens were captured on Nov 27, 2009 and Dec 2, 2008. The earliest capture of *C. obsoletus* was on Mar 25, 2010. In 2009/2010, traps ran continuously during the whole winter at the five selected cattle localities, but no *Culicoides* were found.

Parity dynamics of *Culicoides obsoletus*

At five selected livestock sampling sites (Nos. 2, 11, 18, 20, and 23) and at all hoofed game localities, the physiological status of *Culicoides obsoletus* females were analyzed according to (Dyce 1969) to determine the parity rate dynamics over the five studied years from 2008 to 2013. The rate of parity and the proportion of females with eggs were similar in both types of studied localities (Fig. 5). In livestock farms, where biting midges were captured throughout most of the year, the proportion of parous females was lower in the beginning of the year and the first peak appeared at the end of May. Later, parous females were present in a relatively high proportion till the end of the year, 31.7% in average (Fig. 5A). Overall, the highest percentage of parous females ever recorded was 78.3% in mid-July of 2013. Similarly as at livestock farms, at localities with hoofed game the proportion of parous females was relatively high and stable throughout the year, 39.0 % in average, and since we caught midges just during three months of the year, the proportion of parous females only varied between 18.8 % and 63.2 % (Fig. 5B).

Discussion

Species composition

During the past few decades, several emerging or re-emerging infection diseases transmitted by blood-sucking arthropods have been recorded in Central Europe (e.g. Hubálek 2008, Votýpka et al. 2008), including changes of vector and pathogen spatial distributions and shifting to higher altitudes (Daniel et al. 2003). Biting midges of the genus *Culicoides* are frequently studied as an important subject of human and veterinary medicines, and public health interest has markedly increased in recent years in relation to outbreaks of transmitted viral diseases, especially the bluetongue and Schmallenberg viruses. We have studied the occurrence, seasonal dynamics and proportion of parous females of *Culicoides* species at two types of localities, in the proximity of livestock farms and hoofed game preserves, in the Czech Republic over six years, from 2008 to 2013.

Previously, 63 species had been recorded from the former Czechoslovakia (Országh 1980), with 49 species in the Czech Republic and 55 species in Slovakia (Tóthová and Knoz 2009). Recently, five new species were added and the checklist of *Culicoides* species found in SK has been updated to 63 species (Sarvašová, Kočišová, et al. 2014). Our study updates the number of *Culicoides* species in the Czech Republic to the current 52 species by the adding of three new species: *Culicoides clastrieri*, *C. odiatus*, and *C. saevus*. All these three new species of biting midges are thermophilic species that have recently been found in Slovakia (Tóthová and Knoz 2009, Sarvašová, Kočišová, et al. 2014), and their occurrence in the Czech Republic can be linked to global climate changes, as described previously for other hematophagous diptera (Wittmann et al. 2001, Votýpka et al. 2008). In Bohemia, we found for the first time seven species that were previously known just from Moravia: *C. salinarius* Kieffer, *C. riethi* Kieffer, *C. dzhafarovi* Remm, *C. furcillatus*, *C. pseudoheliophilus* Callot et Kremer, *C. simulator* Edwards, and *C. tauricus* Gutsevich,; and vice versa the species *C. abchazicus* Dzhafarov was found for the first time in Moravia.

In most studies published after 1980, mainly faunistic, trapping sites were selected in wooded fields, meadows and nearby waterways using different collecting methods like light traps, Malaise traps and entomological nets, including untraditionally a net attached to the hood of a car (Knoz 1998, Tóthová et al. 2005). Our study, in contrast, focused especially on biting midges of veterinary and medical public health importance – potential vectors of bluetongue or Schmallenberg viruses – and the choice of localities and trapping method were adapted to this focus. It is likely that our choice of backlight trapping methods (see Venter et al. 2009, De Regge et al. 2015) in combination with the presence of domestic or wild animals close to traps significantly influenced the spectra of *Culicoides* found. We assume that a significant factor in the occurrence of biting midges at our monitored localities was notably the presence of host animals, large ungulates. This ensured that the environment was hospitable enough for biting midges, and other environmental factors, e.g. altitude, had no significant effect on the incidence and abundance of biting midge species.

The majority of *Culicoides* species caught during our study are considered mammaliophilic. The most abundant and widespread species was the *Culicoides obsoletus* complex, regularly representing about 80% of the catches. This species was also the most abundant in other central European countries where similar surveillances were carried out (Linto et al. 2002, De Liberato et al. 2003, Clausen et al. 2009, Ander et al. 2012, De Regge et al. 2015). The *Culicoides obsoletus* complex consists of four species (Conte et al. 2003, Meiswinkel et al. 2004), out of which three were found in our study. The presence of the fourth species, *C. scoticus*, was not detected, though this species was previously described from our territory (Tóthová and Knoz 2009). Different catching methods have been used in other studies, however, and this species was usually collected in very low numbers. As we used multiplex PCR for identification of the *C. obsoletus* complex using only a few specimens from each locality, the (apparent) absence of *C. scoticus* in our samples could be explained by its very low proportion/abundance. It is well known that the *C. obsoletus* complex together with two members of the *pulicaris* complex, *C. pulicaris* and *C. punctatus*, are widespread in Northern and Central Europe (Dijkstra et al. 2008, Hoffmann et al. 2009). The females of species included in the *pulicaris* and *obsoletus* complexes preferably attack livestock (Bartsch et al. 2009, Ninio et al. 2011), and therefore may play a role in the transmission of the bluetongue and Schmallenberg viruses.

Domesticated livestock vs. hoofed game

It is well known that causative agents of several diseases are shared between wildlife and livestock, among them the bluetongue virus (Gortázar et al. 2007), and that pathogens can be easily overlooked when only circulating in wild animals (García et al. 2009). In this study, we compared biting midge faunas between two types of localities, livestock vs. hoofed game. Several *Culicoides* species were common for both types of localities, including *C. obsoletus*, a proven vector of the bluetongue and Schmallenberg viruses. On the other hand, a clear difference was found in the total species number caught at localities with livestock (26 species) versus hoofed games (16 species). This discrepancy can be explained by the different number of trap/nights, which was significantly lower for hoofed game (147) compared to livestock (3,443). This is because localities with livestock were monitored throughout much of the year, from early spring till late autumn or early winter, while localities with hoofed game were sampled only for three months (June to September). In addition, the numbers of captured specimens and notably species composition at sites with hoofed game could be influenced by the surrounding environment (e.g. forest) and the non-continuous presence of host ungulates (in contrast to farms where livestock were present in stables almost continuously in the close proximity of traps). For example, the higher proportion of ornithophilic species, e.g. *C. circumscriptus*, *C. reconditus* and *C. festivipennis* (Országh 1980), in hoofed game traps was probably influenced by the surrounding forest environment that accommodates additional hosts. Similarly, the relatively high proportion (2.8%) of *Culicoides furcillatus* in hoofed game traps is in accordance with previous findings of this sylvatic species (Országh 1980) in a forest habitat in Spain collected by Monteys et al (2009).

Seasonal dynamics of *Culicoides obsoletus*

The seasonal dynamics of the most abundant species, *Culicoides obsoletus* complex, reaching more than 90% of all biting midges in some samples, was analyzed at five selected livestock localities. Several peaks in *C. obsoletus* abundance suggest several consecutives generations during the year and indicate that *C. obsoletus* is a multivoltine species, as described earlier (Országh 1980, Meiswinkel et al. 2014). The first and usually highest peak appeared in the second half of May, as the first generation emerged. Later in the year, several less evident and not well-synchronized peaks appear. The same tendency was also observed in hoofed game samples, even though we do not have data from most of the year. The earliest capture of *C. obsoletus* (and any *Culicoides* species in general) was on Apr 8 (2010) and the last on Dec 2 (2008). According to these data, the biting midge-free (vector-free) period lasts approximately from December till March. The seasonal dynamics of insect populations including biting midges is mainly driven by climatic factors, particularly by rain and temperature (Venail et al. 2012), furthermore by air humidity, light intensity and wind speed (Carpenter et al. 2008). For example in Sweden, the first *Culicoides* midges emerge in May to June, depending on locality, and are last caught in August to September (Ander et al. 2012). On the contrary, in Belgium the vector free period is limited to just January (De Regge et al. 2015).

Parity dynamics of *Culicoides obsoletus*

If transovarial transmission is not considered, only parous females that already fed one or more times can be a vector and are able to transmit pathogens (and thus infections) to their vertebrate hosts. This is the main reason why the knowledge of parity status is so important for epidemiological and ecological studies. In our study we found that the proportion of parous females in the studied population of biting midges at both types of localities was relatively high, fluctuating between 30 to 40% in average. Parity rates varied during the year at both livestock and hoofed game localities, as *Culicoides obsoletus* is a multivoltine species with more generations per year; its parity rate decreased with the disappearance of the old generation and then increased again with feeding of the new generation. Just a few studies have focused on seasonal dynamics of parous biting midge females. In California, *Culicoides vairiipennis* tend to have a highest proportion of nulliparous females in the early season and later the proportion fluctuated around 50% (Linhares and Anderson 1989) and also in Virginia the proportion of *C. obsoletus* parous females varied throughout the seasons (Zimmermann 1983). In the Slovak republic, in the virological study *C. obsoletus* parous females ranged from 11 to 80% in May/June catches (Sarvašová, Goffredo, et al. 2014).

The overall parity rate was higher in hoofed game localities, where host abundance and concentration was lower compare to livestock localities. It should be noted, however, that at game localities we did not sample during the spring and autumn seasons, and in early spring the parity rate seems to be notably lower than during the rest of the summer and autumn. But even if comparing the overlapping part of year when both livestock and hoofed game localities were sampled, parity rates remained slightly higher near hoofed game.

In conclusion, a relatively high number of *Culicoides* species occurs in close proximity to livestock stables and likely feed on their inhabitants. Some of these species are also present at hoofed game localities and could serve as bridge vectors for pathogens shared by both groups of ungulates. Although we found three new thermophilic biting midge species in the Czech Republic, our study yielded no evidence that African and South European BTV vector *C. imicola* midges occur in the country. Therefore, the transmission of bluetongue and Schmallenberg viruses is likely linked to local *Culicoides* species, as in other European countries (Meiswinkel et al. 2007, Dijkstra et al. 2008, Hoffmann et al. 2009). Further host preferences and virological analysis would be beneficial to determine vector species in the Czech Republic.

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Table 1. List of livestock catching sites

No	Name	GPS-N	GPS-E	alt.	No	Name	GPS-N	GPS-E	alt.
1	Dlouhý Újezd	49°46'07"	12°38'08"	520	18	Hradištko	49°51'44"	14°24'59"	286
2	Předslav	49°26'25"	13°21'09"	407	19	Středmy	50°23'01"	14°33'22"	250
3	Kralovice	49°58'02"	13°27'15"	465	20	Zákupy	50°42'32"	14°37'07"	316
4	Lučina	50°13'54"	13°00'47"	471	21	Olešná	49°32'43"	16°07´19"	608
5	Havlíčkův Brod	49°36'40"	15°33'09"	412	22	Markvarec	49°5'2"	15°21'15"	537
6	Dlouhá Ves	50°25'56"	15°47'18"	310	23	Rantířov	49°24'33"	15°30'50"	491
7	Pracejovice	49°13'18"	13°47'48"	627	24	Věcov	49°36'50	16°10'17	619
8	Křižanovice	49°51'39"	15°45'46"	487	25	Těšany	49°2'56"	16°46'3"	209
9	Dvůr Králové	50°26'13"	15°48'24"	304	26	Koroseky	48°55'54"	14°23'34"	438
10	Srbská Kamenice	50°48'51"	14°21'09"	220	27	Krčmaň	49°31'13"	17°19'53"	215
11	Zásmuky	49°58'10"	15°01'54"	307	28	Těšov	49°1'33	17°40'50	232
12	Úpořiny	50°36'52"	13°52'24"	224	29	Heřmanovice	50°10'28"	17°25'30"	568
13	Dmýšice	49°31'30"	14°19'54"	546	30	Březí	48°48'47"	16°34'22"	193
14	Dolní Žandov	49°54'52"	12°40'40"	542	31	Skorošice	50°19'1"	17°01'1"	537
15	Struhařov	49°45'52"	14°45'22"	422	32	Trnava u Zlína	49°16'51"	17°49'20"	346
16	Roudný	50°33'02"	15°16'12"	326	33	Skalice	49°39'38.	18°23'46	345
17	Ruda	50°08'27"	13°52'32"	418	34	Osík u Litomyšle	49°50'16"	16°16'18"	385

Table 2. List of hoofed game sampling sites

No	name	GPS-N	GPS-E	altitude	animals
1	Běléčko	50°9'10"	15°57'41"	287	mouflon, fallow deer
2	Břevnice	49°39'12"	15°36'59"	471	red deer, fallow deer
3	Lázně Bohdaneč	50°5'2"	15°39'52"	222	mouflon, fallow deer
4	Vřísek	50°36'44"	14°30'29"	304	mouflon, bezoar goat
5	Žleby	49°53'2"	15°29'5"	245	red deer, fallow deer, sika deer, Cameroon goat, mouflon
6	Bystrá	50°36'4"	15°24'3"	474	fallow deer
7A	Vrchlabí	50°37'32"	15°37'46"	504	red deer, fallow deer, mouflon
7B	KRNAP	50°41'24"	15°36'26"	974	red deer

Table 3. Biting midge species captured in CDC traps situated in the proximity of livestock and hoofed game.

Culicoides	**livestock**		**game**		***Culicoides***	**livestock**		**game**	
Subgenera/species	No.	%	No.	%	***Subgenera/species***	No.	%	No.	%
Oecacta					***Avaritia***				
achrayi	36	0.01			*abchazicus*	193	0.04		
clastrieri	42	0.01	96	0.54	*obsoletus* complex	408856	91.04	9287	52.30
pallidicornis	4525	1.1	3336	18.79	***Beltranmyia***				
dzhafarovi	172	0.04			*circumsriptus*	736	0.16	256	1.44
fascipennis	4	0.00	14	0.08	*salinarius*	10	0.00		
festivipennis	724	0.16	267	1.50	***Culicoides***				
furcillatus	20	0.00	497	2.80	*grisescens*	180	0.04		
heliophilus	3329	0.74	113	0.64	*impunctatus*	204	0.05	35	0.20
jurensis	8	0.00			*pulicaris*	14955	3.33	1312	7.39
cubitalis	2	0.00	113	0.64	*punctatus*	10138	2.26	710	4.00
minutissimus	23	0.01			***Monoculicoides***				
odiatus	1406	0.31			*nubeculosus*	1960	0.44	892	5.02
pictipennis	452	0.10	152	0.86	*riethi*	11	0.00		
seudoheliophilus	20	0.00			*stigma*	6	0.00	2	0.01
reconditus	563	0.13	514	2.89	***Ponctoculicoides***				
segnis	221	0.05	15	0.08	*tauricus*	68	0.02		
simulator	2	0.00			*saevus*	138	0.03	2	0.01
subfasciipennis	3	0.00	111	0.63					
vexans	86	0.02	32	0.18					

Table 4. Presence/absence of *Culicoides* species at sampling sites in close proximity to livestock (1 to 34) and hoofed game (J1 to J7b).

species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	J1	J2	J3	J4	J5	J6	J7a	J7b
C. obsoletus	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
C. pulicaris	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
C. pallidicornis	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
C. punctatus	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	
C. heliophilus	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■		■	■		■	■	■	■	■		■	■	■	■	■	■	■	
C. pictipennis	■	■		■	■	■	■	■		■	■	■	■	■	■	■	■	■	■	■		■			■	■	■		■	■	■	■		■	■	■	■	■	■			
C. nubeculosus	■	■		■		■	■	■			■	■	■	■	■	■	■	■	■	■		■			■	■	■	■	■	■	■		■	■	■	■		■	■	■		
C. reconditus	■	■	■	■	■	■	■		■	■	■	■	■		■					■				■		■	■				■	■	■		■	■	■	■	■	■	■	
C. festivipennis		■			■	■	■	■		■	■	■	■		■	■	■	■		■		■	■	■		■						■			■	■	■	■	■		■	
C. circumscriptus		■		■		■	■	■	■		■	■	■		■		■	■				■			■	■		■		■			■		■	■	■	■	■		■	
C. dzhafarovi	■	■	■	■			■	■			■	■			■	■	■	■		■		■				■	■				■	■										
C. segnis		■	■	■	■	■	■			■		■	■		■					■	■			■			■					■							■	■	■	
C. impunctatus	■				■					■		■				■	■	■	■			■	■	■		■					■				■	■		■		■		
C. odiatus			■	■	■	■	■				■	■	■		■		■			■				■								■										
C. furcilatus		■					■				■		■		■																				■	■	■	■	■	■		
C. achrayi					■		■	■			■									■	■	■	■	■																		
C. saevus			■					■				■			■	■	■	■														■								■		
C. pumilus	■				■	■	■					■			■									■																		
C. clastrieri		■				■						■										■										■								■		
C. stigma		■						■			■													■		■										■	■					
C. cubitalis																										■									■	■		■	■			
C. subfascipennis															■											■									■			■		■		
C. vexans						■	■				■	■																									■		■			
C. abchazicus	■											■					■															■										
C. fascipennis																	■														■				■	■						
C. jurensis		■		■								■																				■										
C. tauricus				■													■																■									
C. riethi								■										■																								
C. grisescens				■																																						
C. pseudoheliophilus																		■																								
C. salinarus																		■																								
C. simulator																														■												

Fig. 1. Distribution map of *Culicoides* sampling sites in the immediate surroundings of livestock (circles) and hoofed game (triangles) in the Czech Republic.

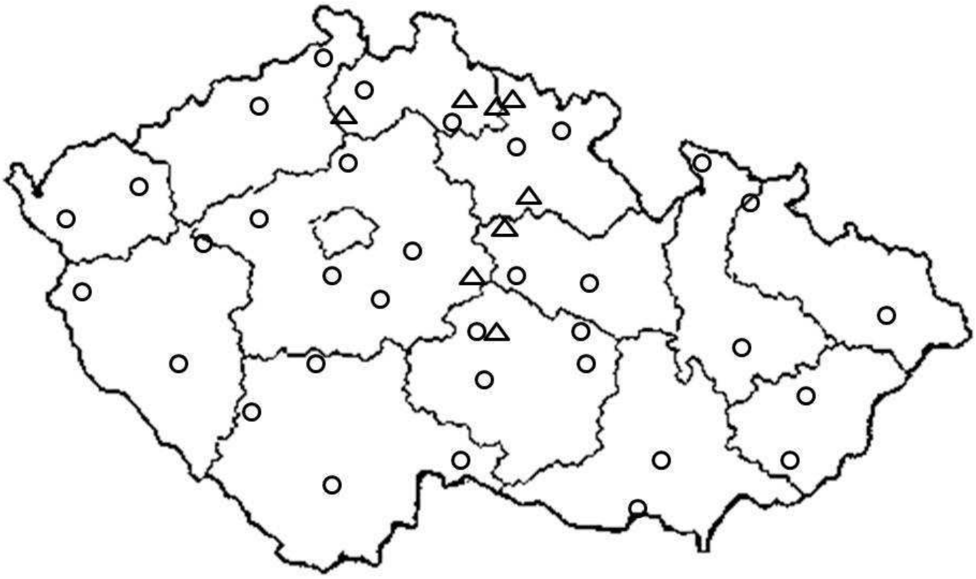


Fig. 2. Proportions of biting midge species captured in CDC traps situated in close proximity to livestock and hoofed game.

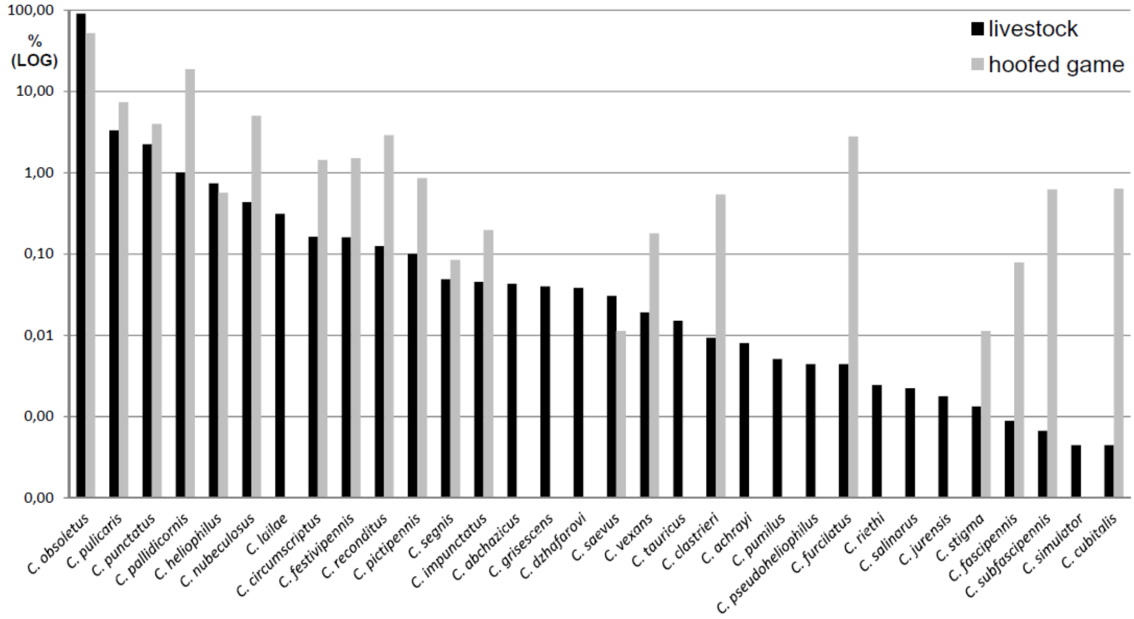


Fig. 3. Linear regression analysis of the altitude of CDC traps, plotted along the Y axis. The total number of *Culicoides* species (A) or specimens (B) is shown along the X axis. The dotted lines show the confidence interval boundaries (95% significance level).

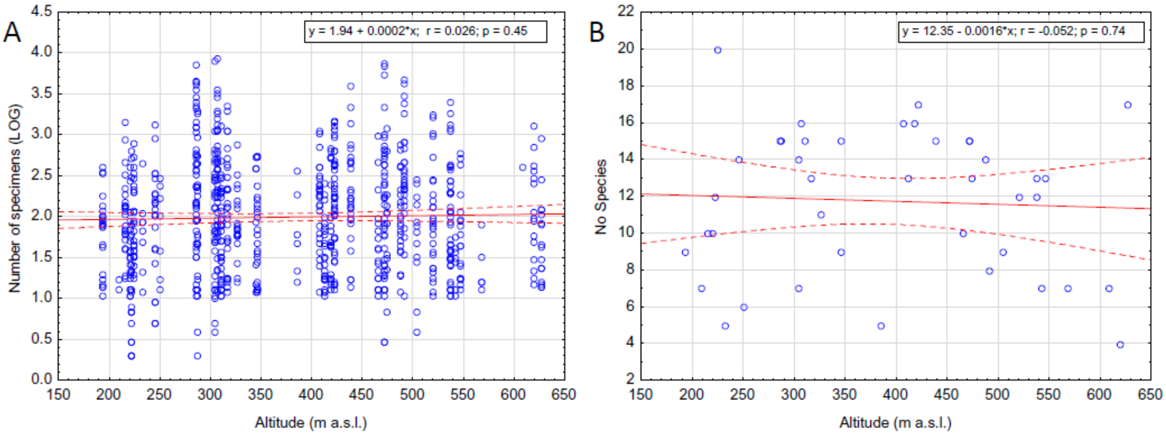


Fig 4. Seasonal dynamics (expressed as the mean of decadal logarithm of captured biting midges per trap) of *Culicoides obsoletus* in livestock localities in four years, 2008 to 2011. april 1 – first two weeks of month, april 2 – second two weeks of month

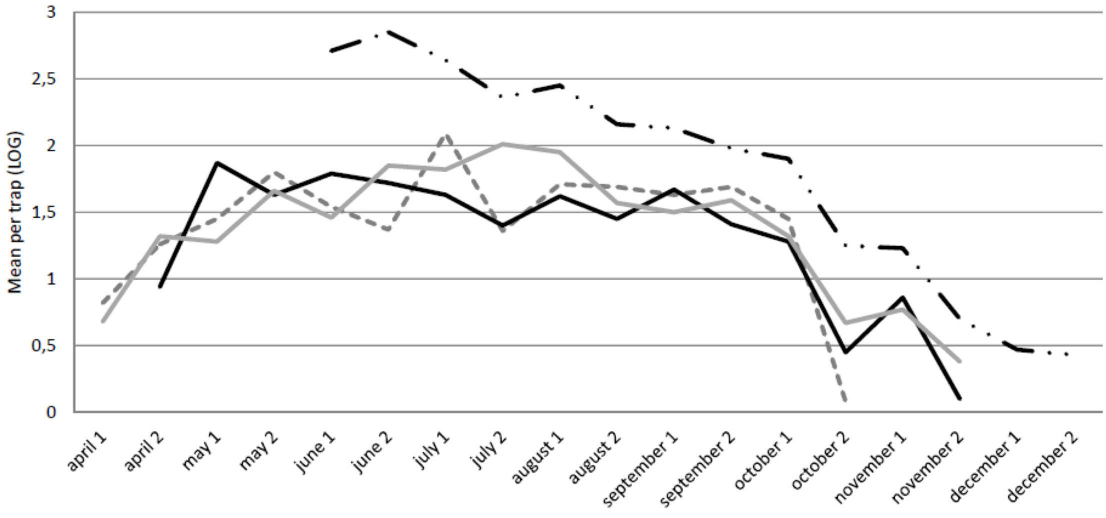
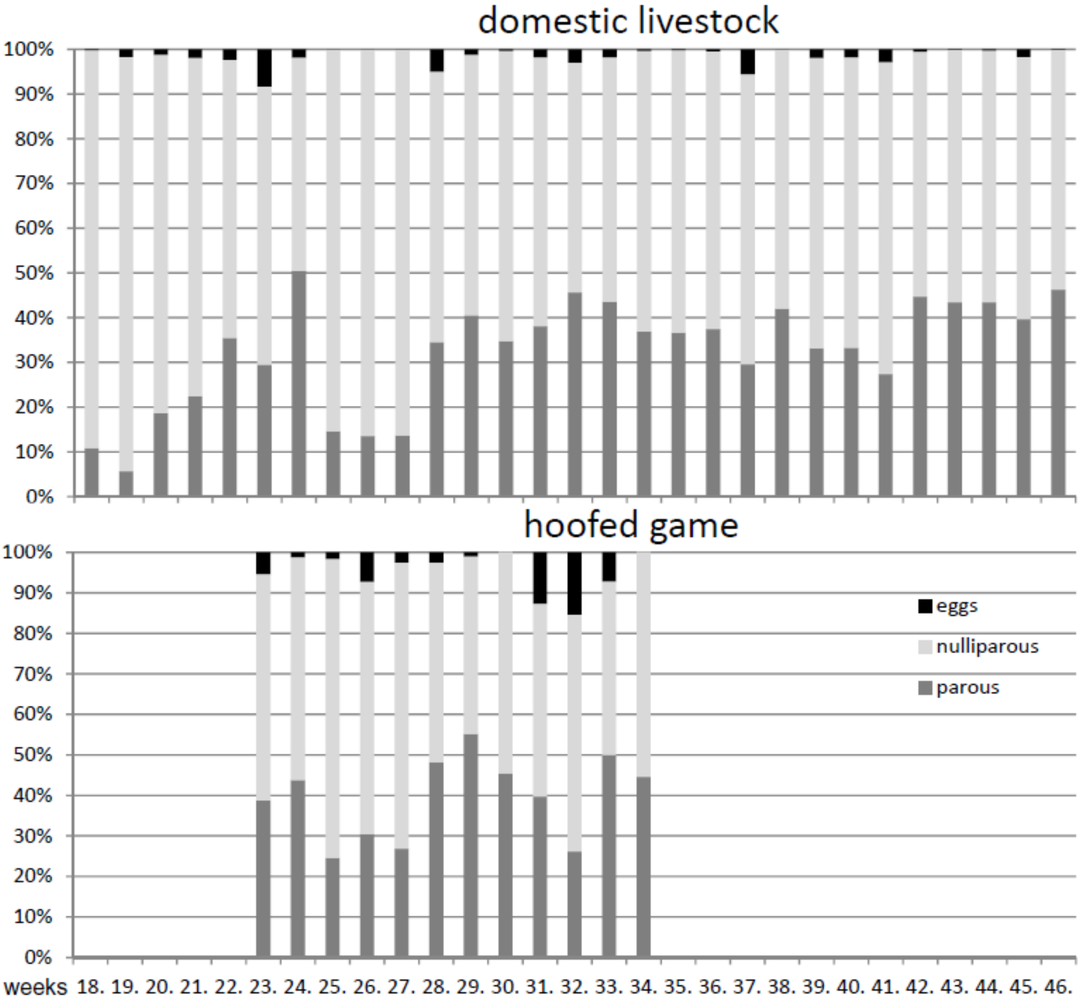


Fig. 5. Combined percentage of parous, nulliparous and gravid females captured in the proximity of livestock farms (A) and hoofed game (B).



Summary

Arthropod-borne diseases infect millions of people annually around the world and mosquito-borne infections are one of the most dangerous. In recent years, several vector-borne diseases affecting humans, domestic and wild animals have (re-) emerged and spread in Europe with major health, ecological, socio-economic and political consequences. The World health organisation estimates that vector-borne diseases account for more than 17% of all infectious diseases, causing more than one million deaths annually, more than 2.5 billion people in over 100 countries are at risk of contracting dengue fever. Malaria causes more than 600.000 deaths every year globally, and other diseases such as Chagas disease, leishmaniasis and schistosomiasis affect hundreds of millions of people worldwide (www.who.int).

Among the arthropod-borne diseases rank the West Nile virus, a human disease with wild birds as natural hosts and Bluetongue or Schmallenberg viruses, important veterinary diseases of ruminants. Two of these three listed pathogens and diseases have been known for years, but have recently been spreading in Europe. Vector-borne viruses in general are introduced into new regions through windborne transportation of insects or with infected hosts transported by international traffic (Barros et al. 2007). Climate changes can also facilitate the expansion of diseases and their vectors to northern localities (Purse et al. 2005, Votýpka et al. 2008, Wilson and Mellor 2008), and bring changes in spatial distribution of vector and pathogen and shifts in altitude was noted (Daniel et al. 2003).

The spreading of vector-borne infections is often linked with the expansion of vectors into new localities as in the example of the tiger mosquitoe *Aedes albopictus* and Dengue (Bonizzoni et al. 2013) or *Phlebotomus* sand flies and *Leishmania* (Melaun et al. 2014). Quite a few studies showed the spread of invasive and thermophilic species of mosquitoes in Europe (Medlock et al. 2012, 2015). A nice example is also the spread of *Culicoides imicola*, originally an afro-tropical species recently spread into the Mediteranean and South Europe (Monteys et al. 2005, Venail et al. 2012). New faunistic studies will elucidate new species speeding into new localities, perhaps as a consequence of global climate change. Similarly, presence of the thermophilous species *Culex martini*, *Uranotaenia unguiculata* and *Anopheles hyrcanus* can be related to the presumed climatic changes (Votýpka et al. 2008, Šebesta et al. 2012). In our studies, we have shown that the appearance of

new species is still a current topic. During entomologic studies focused on mosquito and biting midge fauna, we recorded four new species of blood sucking Diptera in the territory of the Czech Republic: *Anopheles hyrcanus*, *Culicoides clastrieri, C. odiatus, C. saevus*, moreover *A. hyrcanus* is considered important vector of malaria or Sindbis and Ťahyna viruses. In some studies authors also reveal differences in the species spectrum of mosquitoes at individual study sites and between individual years (Šebesta et al. 2012). Not only the species spectrum, but also proportions of individual species change between years. *Culex modestus*, earlier described as a rare species (Kramář 1958, Rettich et al. 1978), based on our findings has become quite common in wetland areas in southern, eastern, western and central Bohemia in recent years and it constitutes the major species of mosquito fauna in many wetland localities in our country (Votýpka et al. 2008). Occurrence of the West Nile virus in south Moravia (Hubálek et al. 1998) was confirmed by isolation of the same virus from two mosquito species *Culex pipiens* and *Cx. modestus* (Radrova et al. 2013). Later, another lineage of West Nile virus was isolated and showed to co-circulate in the Czech Republic (Rudolf et al. 2014).

Culex modestus is an important European vector of West Nile virus (Mouchet et al. 1970, Balenghien et al. 2007, Ponçon et al. 2007) and has characteristics of a typical bridge vector for West Nile virus because of its vector competency and ability to feed on mammals and birds. Thus it is considered the main WNV vector (Balenghien et al. 2006). According to our results, based on animal baited traps, *Cx. modestus* is attracted to both bird and mammal hosts equally (Radrova et al. 2013). As a consequence of recent *Cx. modestus* spreading within the Czech Republic and the high willingness to feed on blood from both avian and mammalian hosts, this species seems to be a more appropriate vector of WNV in the Czech Republic. In contrast, *Cx. pipiens* was collected more frequently at bird traps as opposed to mammal-baited traps. However its mammaliophily was confirmed by using animal-baited traps and by sequencing of engorged blood. The mixed host preferences of the Czech *Cx. pipiens* populations support the hypothesis of a north-south gradient of *Cx. pipiens* mosquito host preferences, ranging from strictly ornithophilic populations in the north to mammaliophilic populations in the south (Zimmerman et al. 1988, Jaenson 1990, Braverman et al. 1991). Feeding preferences are therefore an important criterion to determine species as a vector. *Culex pipiens* is probably a vector of WNV between a main bird host, but in some localities can play the role of a bridge vector from birds to humans and other mammals (Hamer et al. 2008, Rádrová et al. 2013).

It is well known that causative agents of several diseases are shared between wildlife and livestock, among them the Bluetongue virus (Gortázar et al. 2007), and that pathogens can be easily overlooked when only circulating in wild animals (García et al. 2009). We compared biting midge fauna between two types of localities, livestock vs. hoofed game. Several *Culicoides* species were common for both types of localities, including *C. obsoletus*, a proven vector of the Bluetongue and Schmallenberg viruses, and again, common species can serve as bridge vectors for infections shared by both hosts. On the other hand, a clear difference was found in the total species number caught at localities with livestock versus hoofed game (Rádrová et al. subm.). Among others, we measured the proportion of parous females in the studied population of biting midges at both types of localities, which was relatively surprisingly high, fluctuating between 30 to 40% on average and varied throughout the seasons, similar to others studies (Zimmerman and Turner 1983, Linhares and Anderson 1989). If transovarial transmission is not considered, only parous females that already fed one or more times can be a vector and are able to transmit pathogens (and thus infections) to their vertebrate hosts.

Bloodsucking insects also transmit exclusively animal diseases, which are sometimes slightly overlooked when they have no veterinary importance. Avian trypanosomes can be transmitted by diverse invertebrates, but their biology (vectors, life cycles, host and vector specificities) is poorly known or totally unknown, despite the fact that some species could be pathogenic to their host (Molyneux et al. 1983). The simple presence of trypanosomes in a blood-sucking invertebrate does not necessarily incriminate it as a vector. Only if parasites survive defecation of the rest of a blood-meal and successfully colonize a digestive tract (or salivary glands) can we speculate about transmission to vertebrate hosts. In our study, we described a novel species of trypanosome from birds, transmitted by *Culex* mosquitoes, *Trypanosoma culicavium,* on the basis of naturally and experimentally infected vectors and bird hosts, localization in the vector, morphological characters and molecular data (Votýpka, et al. 2012).

Bloodsucking diptera are an important group of insects, as they serve as vectors of several diseases. There are many criterions for possible transmission of pathogens, such as the presence in the locality, host preferences of possible vectors, vector competence, vector capacity and other natural conditions. As the climate changes, new species are spreading into new localities, introducing new pathogens. Continuous monitoring and analysis of bloodsucking insects can predict the possible risks of disease outbreaks, and can elucidate unknown or uncertain vector-host relations.

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