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## Host preferences of host-seeking and blood-fed Swiss mosquitoes

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Arbeit unter wissenschaftlicher Betreuung von  
Prof. Dr. sc. nat. Alexander Mathis

## **Host preferences of host-seeking and blood-fed Swiss mosquitoes**

### **Inaugural-Dissertation**

zur Erlangung der Doktorwürde der  
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

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## Inhaltsverzeichnis

Zusammenfassung.....	3
Summary .....	4
<b>Host preferences in host-seeking and blood-fed mosquitoes in Switzerland .....</b>	<b>5</b>
Danksagung	
Curriculum Vitae	

## Zusammenfassung

Das West Nil Virus (WNV) hat sich in den letzten Jahrzehnten in Süd- und Osteuropa ausgebreitet. Hauptwirte des Virus sind Vögel, die gewöhnlich keine klinischen Symptome zeigen. Bei Pferden und Menschen können Infektionen zu neuroinvasiven Krankheiten führen. Stechmücken werden generell als Hauptüberträger (Vektoren) des WNV angesehen. Um das Übertragungsrisiko auf empfängliche Säuger-Wirte einzuschätzen wurden die Wirtspräferenzen von Schweizer Stechmücken mit zwei Methoden untersucht: (1) mittels Tier-Köder Fallen (Pferd und Hühner; unter Berücksichtigung von Körpergewicht, -oberfläche und Metabolismusrate), welche viermal über Nacht zwischen Mai und September 2014 an zwei verschiedenen Standorten (natürlich, periurban) betrieben wurden; (2) mittels Sammlung blutgefütterter Stechmücken im Zoo Zürich und im Feld sowie der molekularbiologischen Identifikation der „Blutspender“. Nach statistischer Analyse der 1058 in Tier-Köder Fallen gefangenen weiblichen und der 566 blutgefütterten Stechmücken wurde bei neun Arten ein opportunistisches Stechverhalten (Blutmahlzeiten sowohl von Vögeln wie auch Säugern) identifiziert, wobei in Blutmahlzeiten der invasiven Buschmücke *Aedes japonicus* erstmals aviäre DNA nachgewiesen wurde. In der Schweiz stellen somit unter Berücksichtigung von Abundanz, räumlich-zeitlicher Aktivität und Vektorkompetenz, neben dem Hauptvektor *Culex pipiens*, *Ae. japonicus* und *Ae. vexans* die besten Brückenvektoren für die Übertragung von WNV in der Schweiz dar.

## Summary

West Nile Virus (WNV) has spread in Southern and Eastern Europe over the last decade. The main hosts for the virus are birds which usually do not show clinical signs. In horses and humans infections can cause neuroinvasive diseases. Mosquitoes are generally considered the main transmitters (vectors) of WNV. To assess the risk for susceptible mammalian hosts, the host preferences of Swiss mosquitoes were investigated with two methods: (1) by means of animal-baited traps (horse and chickens; in consideration of body weight and surface, metabolic rates), which were run four times over night between May and September 2014 at two different sites (natural, periurban); (2) by collecting blood-fed mosquitoes at the Zoo Zürich and in the field and the molecular-biological identification of the 'blood-donors'. Statistical analysis of the 1058 females collected in the animal-baited traps and the 566 blood-fed mosquitoes revealed an opportunistic feeding behaviour (blood meals from birds as well as from mammals) in nine mosquito species whereby for the first time avian DNA was identified in blood meals of the invasive bush mosquito *Aedes japonicus*. Considering abundance, spatio-temporal activity and vector competence, in addition to the main WNV vector *Culex pipiens*, *Ae. japonicus* and *Ae. vexans* represent the best candidate bridge vectors for WNV transmission in Switzerland.

# Host preferences in host-seeking and blood-fed mosquitoes in Switzerland

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**Abstract.** The avian zoonotic agent for West Nile virus (WNV) can cause neuroinvasive disease in horses and humans and is expanding its range in Europe. Analyses of the risk for transmission to these hosts in non-endemic areas are necessary. Host preferences of mosquitoes (Diptera: Culicidae), the main vectors of WNV, were determined in Switzerland using animal-baited trap (horse, chickens) experiments at a natural and a periurban site. This was undertaken on four occasions during May–September 2014. In addition, the hosts of 505 blood-fed mosquitoes collected in a zoo and in the field were determined. Mosquito data obtained in the animal bait experiments were corrected for host weight and body surface area and by Kleiber's scaling factor. Collections of 11–14 different mosquito species were achieved with these approaches. Statistically significant host preferences were identified in three species in both approaches. The other species showed opportunistic feeding behaviours to varying extents. Specifically, the invasive species *Hulecoeteomyia japonica* (= *Aedes japonicus*) was identified for the first time as feeding on avians in nature. Abundance data, spatiotemporal activity and laboratory vector competence for WNV suggested that, in addition to the main WNV vector *Culex pipiens*, *H. japonica* and *Aedimorphus vexans* (= *Aedes vexans*) are the most likely candidate bridge vectors for WNV transmission in Switzerland.

**Key words.** Culicidae, animal-baited trap, body surface area, host weight, Kleiber's scaling factor, natural site, periurban site, West Nile virus, zoo.

## Introduction

West Nile virus (WNV) (Flaviviridae), one of the most frequently reported arboviruses in the world, causes a zoonotic neuroinvasive disease primarily affecting horses and humans (Reiter, 2010). The primary hosts for the virus are birds, which usually do not show clinical signs. However, considerable avian mortality has been observed in Israel and North America (Kilpatrick, 2011). West Nile virus has re-emerged in Europe (Hubálek & Halouzka, 1999) and has expanded its

range by rapidly invading North America (Reisen, 2013). In (south)eastern Europe, the numbers of cases in humans and horses have increased over the last decade and WNV has been reported in new areas. Regularly updated maps of the European distribution of human cases of West Nile fever (WNF) are provided by the European Centre for Disease Prevention and Control (ECDC) (<http://ecdc.europa.eu>). The first human WNV infection in northeast Italy was reported in 2008 (Rossini *et al.*, 2008) and the virus has subsequently been shown to have become endemic and to spread locally (Delbue *et al.*, 2014). In

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2014, the first autochthonous human case of WNV was reported from Vienna, Austria (Jungbauer *et al.*, 2015). The spread of WNV to new areas is thought to occur via the movements of chronically infected migratory birds and long-distance movements of resident birds (Hubálek, 2000; Ciota & Kramer, 2013; Reisen, 2013).

Mosquitoes are generally considered the main biological vectors of WNV. Although the virus has been detected in other haematophagous arthropods (Platonov, 2001), their roles as vectors remain unclear. Further, other modes of transmission between vertebrate hosts (the faecal–oral route or through preying or scavenging on infected animals) are evident but have so far received comparatively little attention (Reiter, 2010). West Nile virus has been isolated from at least 75 mosquito species worldwide. In Europe, two species are implicated as key vectors: *Culex pipiens* (Linnaeus), biotypes *pipiens* and *molestus*, and *Culex modestus* (Ficalbi). However, the virus has been detected in field-collected specimens of an additional 13 species (Hubálek, 2000; Medlock *et al.*, 2005). Experimentally, suitability for pathogen transmission (vector competence) has been shown in the laboratory for a number of species (reviewed by Reisen, 2013). However, the vectorial capacity of a mosquito population (i.e. its efficiency as a vector in the field) depends on several factors other than vector competence. These include host preference, seasonal abundance and longevity (Balenghien *et al.*, 2006; Ciota & Kramer, 2013; Schaffner & Mathis, 2014). Specifically for WNV, transmission from birds to humans or horses requires that mosquitoes take sequential bloodmeals from an infected bird and a susceptible mammalian host, and thus represent bridge vectors (Ciota & Kramer, 2013).

The aim of the present study, which was carried out within the frame of a larger project to address the risk for WNV transmission in Switzerland, was to investigate the host preferences of local mosquito species. This was achieved by: (a) performing host (horse, chicken and human) bait experiments at two sites, a wetland representing a putative site for virus introduction by migratory birds and local enzootic transmission, and a periurban recreational site representing a putative site for the transmission of virus to humans and horses, and (b) bloodmeal analysis of blood-fed mosquitoes collected at the Zürich Zoologischer Garten (Zoo Zürich) and at four field study sites.

## Materials and methods

### Animal-baited trapping

Horse and chicken baits were used in the bait studies. These were conducted in analogy to an earlier study (Balenghien *et al.*, 2006) at two different sites (Fig. 1): a natural site (47°30'22.8" N, 08°28'47.5" E; 414 m a.s.l.), and a periurban recreational site (47°29'54.5" N, 08°39'13.4" E; 644 m a.s.l.). The natural site was located at the edge of a nature reserve (lowland moor, 105 ha) in a small light forest on swampy ground with temporary flooding. The periurban site was located at the edge of a forest.

The experiments were undertaken at each site four times during May–September 2014 (periurban site: 18 and 19 May, 1 and 2 July, 5 and 6 August, 16 and 17 September; natural site: 20

and 21 May, 24 and 25 June, 7 and 8 August, 23 and 24 September). Experiments were scheduled on dates with probable high abundances of local mosquitoes based on the results of mosquito collections by carbon dioxide (CO<sub>2</sub>)-baited Centers for Disease Control (CDC) traps during 2012 and 2013 at these sites (S. Wagner, unpublished data, 2013). An experimental period lasted 12 h and began at 6 h before sunset. Mosquitoes were collected in the animal-baited traps by mouth aspiration and by using a hand-held aspirator (Hausherr's Machine Works, Toms River, NJ, U.S.A.) every 4 h (to differentiate among diurnal, vespertine and nocturnal mosquito species). After each mosquito collection, the other insects in the horse cage were removed by mechanical aspiration with CDC backpack aspirators (John W. Hock Co., Gainesville, FL, U.S.A.).

The horse-baited trap consisted of a metal cage (3 × 3 m), complemented with a wooden frame (height 2.5 m) covered with a net (mesh 40) which protruded from the cage by 70 cm to facilitate the collection of mosquitoes from inside the net without requiring entry to the cage (Fig. 2A). The net was rolled up approximately 40 cm from the ground during the duration of the trial on one side (facing the edge of the grove at the natural site and the open field at the periurban site) to allow mosquitoes to enter the trap. The baiting horse was a 23-year-old Freiberger mare, with a weight of 570 kg. A second horse was present as a companion horse (under a completely closed net) at the study site. Both horses had *ad libitum* access to hay and water.

The bird-baited traps (71 × 61 × 91 cm) were reconstructed from metal dog kennels, which were attached to wooden boards and covered with a net (Fig. 2B). The bottoms of the cages were filled with wood shavings and perches were placed inside. Each bird-baited trap contained two chickens which had *ad libitum* access to food and water. Chickens were obtained from a local breeder. Each experiment included new individuals, each weighing approximately 2 kg, of different breeds and colours. One trap was placed 1 m above the ground and the other 5 m above the ground in the canopy.

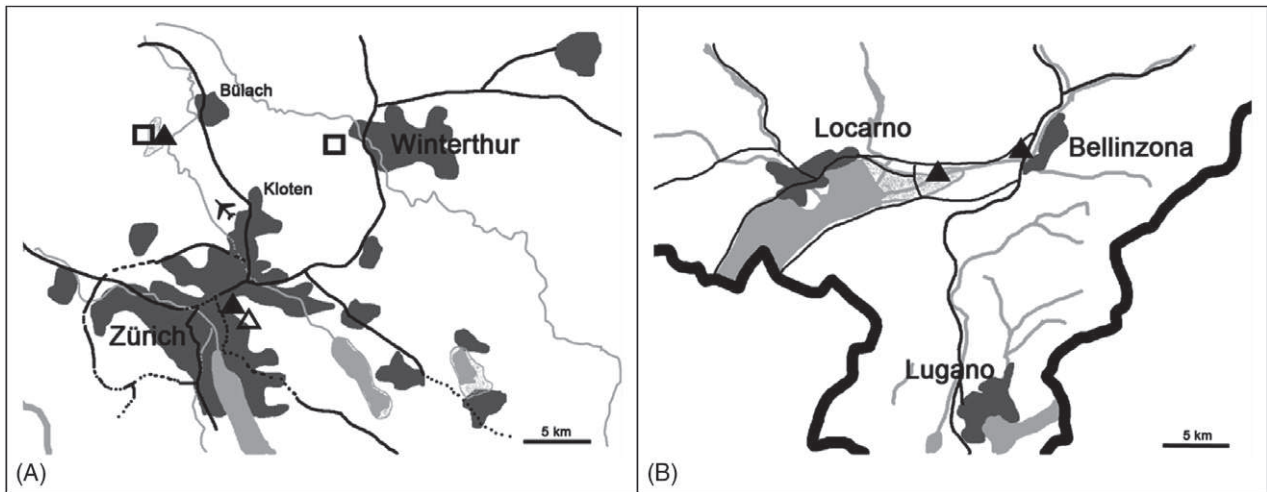
Clinical examinations of both the horses and the chickens were carried out by a veterinarian before the beginning of the trial, with every mosquito collection and upon completion of the experimental period. No abnormal clinical signs were observed throughout the experiments. The study was approved by the Cantonal Veterinary Office of Zurich (permission no. 127/2013).

Mosquitoes landing on humans were collected by aspiration from two or three persons for 15 min once during each study interval (diurnal, vespertine and nocturnal) (Fig. 2C). All persons wore a white overall and exposed a forearm to the mosquitoes.

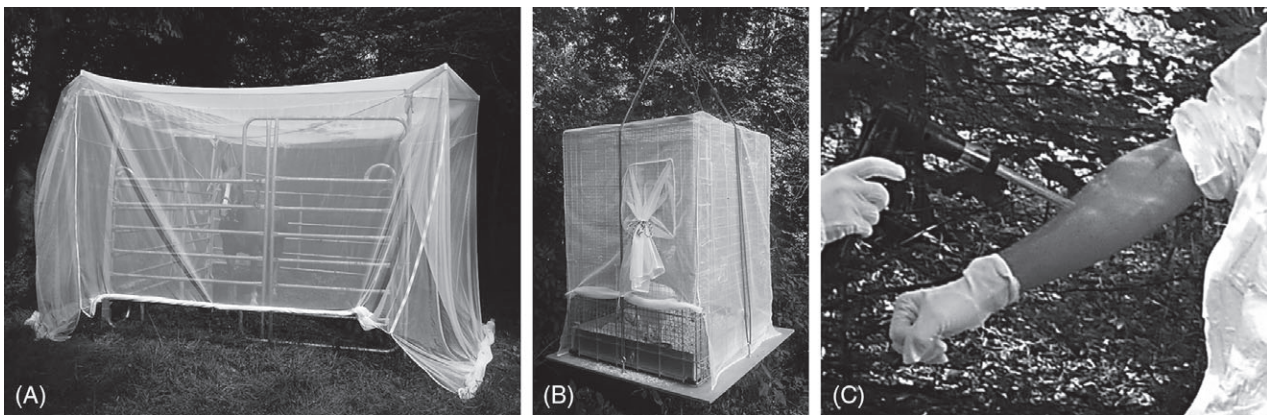
The collected mosquitoes were killed immediately on dry ice and stored at –20 °C until they could be morphologically identified.

### Collection of blood-fed mosquitoes

*Collection at the zoo.* Mosquitoes were collected at Zoo Zürich (www.zoo.ch), Zurich, Switzerland (47°23'06" N, 08°34'23" E) over two consecutive years. The zoo is situated on the outskirts of the city at 610 m a.s.l. and borders a forest. In



**Fig. 1.** Mosquito trapping sites in Switzerland. (A) Area north of the Alpine crest (canton Zurich). (B) Area south of the Alpine crest (canton Ticino). □ indicates sites of animal-baited traps at one natural and one periurban site (2014); △ indicates sites of Zoo Zürich collections of blood-fed mosquitoes using Centers for Disease Control (CDC)-iGu<sup>®</sup> traps, gravid traps and aspirator (2013, 2014); ▲ indicates sites of collection of blood-fed mosquitoes by CDC-iGu<sup>®</sup> traps at one natural and one periurban site during 2012–2014. Dark grey-shaded areas represent residential areas/cities; light grey-shaded areas indicate waters; the thick black line shows the national border.



**Fig. 2.** (A) Horse-baited trap. (B) Chicken-baited trap. (C) Retrieval of human landings.

2013, mosquitoes were collected between July and October at eight different sites, once per month over 1 night. Two different mosquito trap types were used at each site. These included the CO<sub>2</sub>-iGu trap, a CDC miniature light trap (Model 1012; John W. Hock Co.) baited with dry ice and a pheromone dispenser using 1-octen-3-ol and ammonium bicarbonate (iGu<sup>®</sup> Combi FRC 3003; Silva GmbH & Co. KG, Lübeck, Germany), and either of two gravid traps, the Reiter Gravid Mosquito Trap (Model 2800; BioQuip Products, Inc., Rancho Dominguez, CA, U.S.A.) or the Frommer Updraft Gravid Trap (Model 1719; John W. Hock Co.). The gravid traps contained an oak leaf infusion (4.2 g of oak leaves per litre of water, incubated at 27 °C for 7 days). The collection sites were selected according to characteristics described in Tuten (2011). In addition, mosquitoes were collected with a hand-held aspirator once per week. In 2014, blood-fed mosquitoes were collected by aspiration only, once per week from April until October. At the beginning of the

study, 12 sites were selected for the mechanical collection of mosquitoes and new sites were added continuously during the course of the study on an ad hoc basis.

*Collection in the field.* Blood-fed mosquitoes were also trapped in the field during a parallel study (S. Wagner, unpublished data, 2014). In brief, mosquitoes were collected at a natural and a periurban site in the vicinity of an extended wetland on the northern and southern sides of the Alpine Crest (north of the Alps: 47°23'49" N, 08°33'14" E; 550 m a.s.l.; 47°30'23" N, 08°28'48" E; 414 m a.s.l.; south of the Alps: 46°11'14" N, 08°59'30" E; 230 m a.s.l.; 46°09'46" N, 08°54'14" E; 200 m a.s.l.) (Fig. 1). Carbon dioxide-baited CDC traps in 2012 and CO<sub>2</sub>-iGu CDC traps in 2013 biweekly, over two consecutive nights, were used. Additionally, blood-engorged females were collected with CDC backpack aspirators and by netting



(collapsible insect nets; Bioform Entomology & Equipment, Nürnberg, Germany) at both sites in September 2012. Tree trunks, tree holes, piles of wood stock or garden waste, lower vegetation, solid fences and walls, and ground depressions or ditches along ecotones at the forest border were examined as potential resting sites. Additional traps were set up over three consecutive nights at the site north of the Alps in October 2012. These included two gravid traps, four resting traps (BioQuip Products, Inc.), and two BG-Sentinel™ (BGS) traps and two BG-Mosquitaire™ (BGM) traps, each baited with BG-Sweetscent™ (Biogents AG, Regensburg, Germany).

#### *Mosquito identification*

All mosquitoes were morphologically identified to species or sister taxa level using different keys (Schaffner *et al.*, 2001; Becker *et al.*, 2010). Blood-fed mosquitoes collected at Zoo Zürich were also grouped according to Sella stage (Tuten *et al.*, 2012).

*Culex pipiens* and *Culex torrentium* (Martini) were differentiated by species-specific polymerase chain reaction (PCR) targeting the acetylcholinesterase-2 gene as described by Smith & Fonseca (2004). The head and thorax of individual insects were ground in 180 µL Tris-EDTA buffer (pH 8.4) using a mixer mill (MM 300; Retsch GmbH, Haan, Germany) with one steel bead (3 mm in diameter) at 30 Hz for 1 min twice with a centrifugation step between grindings (Wenk *et al.*, 2012). DNA was isolated from pools of homogenates (10 µL from each individual, maximum 18 insects per pool) using a commercial kit (Qiagen DNA Mini Kit; Qiagen GmbH, Hildesheim, Germany). DNA was eluted with 55 µL of water, and PCRs with 1 µL of the elutes were performed in a thermal cycler (DNA engine; MJ Research, Bio-Rad Laboratories, Basel, Switzerland) as described previously (Trachsel *et al.*, 2007). The uracil DNA glycosylase system (Fisher Scientific AG, Reinach, Switzerland) was used to prevent carryover contamination. Amplification products were run on a 1.5% agarose gel stained with GelRed™ (Biotium, Inc., Hayward, CA, U.S.A.) and were visualized by ultraviolet (UV) transillumination.

#### *Bloodmeal analysis: DNA isolation; PCR; sequencing, and cloning*

The abdomens of blood-fed mosquitoes, collected at the zoo, were separated from the head and thorax with a razor blade (cleaned with 70% EtOH and flame-sterilized after each mosquito) and stored separately in 95% EtOH in 2-mL Eppendorf tubes (Vaudaux-Eppendorf AG, Schönenbuch, Switzerland) at 4 °C. Separated abdomens from the field-collected mosquitoes were stored in 70% EtOH at –20 °C.

Abdomens were ground individually, as described above, in 180 µL Tris-EDTA buffer (pH 8.4). The homogenate was then incubated in a heating block at 95 °C for 5 min, and DNA was isolated using the QIAamp DNA Mini Kit (Qiagen GmbH) according to the manufacturer's instructions for blood. DNA was eluted in 55 µL AE buffer and stored at –20 °C until further use.

DNA extracts from mosquito abdomens were amplified as described above, using 5 µL isolated DNA and primers targeting the mitochondrial cytochrome b gene that were designed to be vertebrate-specific [L14841/H15149 (Kocher *et al.*, 1989), Cytb (f)/Cytb (r) (Townzen *et al.*, 2008)] or class-specific [mammal, avian (Ngo & Kramer, 2003)].

Polymerase chain reaction products were purified with the Minelute PCR Purification Kit (Qiagen GmbH). DNA concentration was measured using a NanoDrop® 1000 photometer (NanoDrop Products, Thermo Fisher Scientific, Inc., Wilmington, DE, U.S.A.) and diluted to 6 ng/µL. Sequencing was undertaken by a private company (Synergene GmbH, Schlieren, Switzerland). The quality of the sequences was assessed using FinchTV (www.geospiza.com), and the blood host species was identified through comparison with the GenBank DNA database using BLAST (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). Findings were considered reliable if the percentage identity with a GenBank entry was higher than 95% and the host species identified was known to occur in the neighbourhood of the collection site.

Amplicons from six samples that yielded multiple overlapping peaks in the electropherogrammes were cloned into the Topo-TA-cloning vector according to the manufacturer's instructions (Invitrogen, Inc., Carlsbad, CA, U.S.A.). Four clones of each were sequenced.

#### *Statistical analysis*

Mosquito data obtained in the animal bait experiments were corrected by host body weight (BW), body surface area (BSA) (Stahl, 1967) and Kleiber's scaling factor (KSF) (Kleiber, 1947). Body weights of the four chickens included in the experiments were considered as one 'chicken mass'.

Differences in the numbers of mosquitoes recovered from each trap were analysed on the assumption that they followed a Poisson process. The numbers of mosquitoes recovered from a single trap were assumed to be directly proportional to the BW, BSA or KSF of the bait species in the trap. Differences in mosquito numbers adjusted for the size of the bait species were analysed using Poisson rate ratios. A rate ratio of 1 indicates that the same number of mosquitoes was recovered. Thus, if the confidence limit of the Poisson rate ratio does not include 1, it can be concluded that significantly different numbers of mosquitoes were recovered. All calculations were undertaken in R (R Core Team, 2015), using the package 'exactci'. To determine statistically significant differences in the host preferences of mosquitoes collected at Zoo Zürich and in the field, respectively, 95% Poisson confidence intervals (CIs) were calculated in R.

## **Results**

### *Animal-baited trapping*

*Diversity of the mosquito species collected.* Collections amounted to a total of 899 female and eight male mosquitoes

**Table 1.** Diversity and abundance of mosquito species in host-baited collections at a natural and a periurban site in Switzerland (cumulative numbers from four trapping events during May–September 2014)

Bait	Natural site		Suburban site			
	Mosquito species	Females, <i>n</i> (engorged, <i>n</i> )	Males, <i>n</i>	Mosquito species	Females, <i>n</i> (engorged, <i>n</i> )	Males, <i>n</i>
Horse	<i>Anopheles claviger</i>	319 (250)	2	<i>Ochlerotatus cantans/annulipes</i>	82 (56)	2
	<i>Ochlerotatus cantans/annulipes</i>	150 (116)	4	<i>Ochlerotatus rusticus</i>	34 (28)	0
	<i>Aedimorphus vexans</i>	107 (98)	0	<i>Hulecoeteomyia japonica</i>	19 (16)	0
	<i>Coquillettidia richiardii</i>	83 (58)	0	<i>Aedes cinereus/geminus</i>	14 (10)	0
	<i>Anopheles maculipennis s.l.</i>	81 (28)	1	<i>Anopheles claviger</i>	5 (1)	0
	<i>Culiseta annulata</i>	30 (17)	0	<i>Coquillettidia richiardii</i>	4 (4)	0
	<i>Aedes cinereus/geminus</i>	20 (12)	0	<i>Aedes species*</i>	3 (3)	0
	<i>Culex pipiens</i>	20 (0)	0	<i>Culex pipiens</i>	3 (0)	0
	<i>Hulecoeteomyia japonica</i>	8 (6)	0	<i>Aedimorphus vexans</i>	2 (2)	0
	<i>Ochlerotatus sticticus</i>	8 (8)	0	<i>Ochlerotatus cataphylla</i>	1 (0)	0
	<i>Culiseta morsitans</i>	1 (0)	1	<i>Dahlia geniculata</i>	1 (1)	0
	<i>Dahlia geniculata</i>	1 (1)	0	<i>Culiseta annulata</i>	1 (0)	0
	<i>Ochlerotatus rusticus</i>	1 (1)	0			
	<i>Culex territans</i>	1 (0)	0			
Chicken (cage 1 m above ground)	<i>Coquillettidia richiardii</i>	11 (3)	0	<i>Culex pipiens</i>	4 (1)	0
	<i>Culex pipiens</i>	6 (4)	0			
	<i>Culex</i> or <i>Coquillettidia</i> species†	3 (0)	0			
	<i>Ochlerotatus cantans/annulipes</i>	1 (0)	0			
	<i>Aedes cinereus/geminus</i>	1 (0)	0			
	<i>Aedimorphus vexans</i>	1 (0)	0			
Chicken (cage ~5 m above ground)	<i>Culiseta annulata</i>	1 (0)	0			
	<i>Culex pipiens</i>	19 (10)	0	<i>Culex pipiens</i>	3 (0)	0
	<i>Coquillettidia richiardii</i>	6 (2)	0			
	<i>Ochlerotatus cantans/annulipes</i>	1 (0)	0			
Human	<i>Aedimorphus vexans</i>	1 (0)	0			
	<i>Ochlerotatus cantans/annulipes</i>	11 (0)	0	<i>Ochlerotatus cantans/annulipes</i>	3 (0)	0
	<i>Coquillettidia richiardii</i>	6 (0)	0	<i>Hulecoeteomyia japonica</i>	1 (0)	0
Total	<i>Aedes cinereus/geminus</i>	1 (0)	0	<i>Coquillettidia richiardii</i>	1 (0)	0
		899 (614)	8		181 (122)	2

\*Damaged specimens of the genus *Aedes* that could not be further identified.

†Mosquitoes with a round abdomen that escaped during field work.

of 14 different species at the natural site, and 182 female and two male mosquitoes of 11 different species at the suburban site during the four trapping events (Table 1). The horse baited 1000, the four chickens 58, and humans 23 female mosquito specimens.

The 55 *Cx. pipiens/torrentium* collected by animal baiting (Table 1) were subjected to species-specific PCRs in pools, all of which were positive for *Cx. pipiens* and negative for *Cx. torrentium*.

At the natural site, *Anopheles claviger* (Meigen), *Ochlerotatus cantans/annulipes* (= *Aedes cantans/annulipes*) (Meigen/Walker), *Aedimorphus vexans* (= *Aedes vexans*) (Meigen), *Coquillettidia richiardii* (Ficalbi), *Anopheles maculipennis s.l.* (Meigen), *Culiseta annulata* (Schränk), *Aedes cinereus/geminus* (Theobald/Peus) and *Cx. pipiens* represented 97.6% of all female mosquitoes collected in the horse-baited trap (Table 1), whereas *Cx. pipiens* and *Cq. richiardii* represented 88.2% of mosquitoes collected in the bird-baited traps. Twenty-four mosquitoes were collected in the cage close to the ground, with *Cq. richiardii* being the most frequent species, and 27 mosquitoes were retrieved

from the cage in the canopy, mainly comprising *Cx. pipiens*. Human landings yielded 18 mosquitoes of three species (*O. cantans/annulipes*, *Cq. richiardii* and *Ae. cinereus/geminus*), all of which were included among the most frequent species in the horse-baited trap.

At the periurban site, *O. cantans/annulipes*, *Ochlerotatus rusticus* (= *Aedes rusticus*) (Rossi), *Hulecoeteomyia japonica* and *Ae. cinereus/geminus* represented 88.2% of the mosquitoes collected in the horse-baited trap (Table 1). *Culex pipiens* was the only mosquito species collected in the bird-baited traps, in which similar although very small numbers were collected in both the trap on the ground and that in the canopy. All five specimens collected by human landings represented mosquito species also collected in the horse-baited trap (*O. cantans/annulipes*, *H. japonica*, *Cq. richiardii*).

*Host preferences of mosquito species collected.* Seven species were exclusively collected in the horse-baited trap. The majority of these were either *An. claviger* or *An. maculipennis s.l.* No mosquito species was exclusively collected in the

**Table 2.** Corrected abundances of mosquito species in horse- and chicken-baited collections in Switzerland 2014 according to body weight (BW), body surface area (BSA) and Kleiber's scaling factor (KSF)

Mosquito species	Mosquitoes, <i>n</i>		BW*			BSA†			KSF‡		
	Horse	Chicken	Rate ratio	95% CI	<i>P</i> -value	Rate ratio	95% CI	<i>P</i> -value	Rate ratio	95% CI	<i>P</i> -value
<i>Anopheles claviger</i>	326	0	Inf	1.2–Inf	0.02	Inf	8.7–Inf	< 0.0001	Inf	5.1–Inf	< 0.0001
<i>Ochlerotatus cantans/annulipes</i>	239	2	1.67	0.46–13.9	NS	12.1	3.3–100.7	< 0.0001	6.9	1.9–57.2	0.0003
<i>Aedimorphus vexans</i>	109	2	0.7	0.2–6.4	NS	5.5	1.5–46.2	0.003	3.1	0.8–26.2	NS
<i>Coquillettidia richiardii</i>	87	17	0.07	0.04–0.12	< 0.0001	0.5	0.3–0.9	0.02	0.29	0.17–0.53	< 0.0001
<i>Anopheles maculipennis s.l.</i>	82	0	Inf	0.3–Inf	NS	Inf	2.2–Inf	0.0007	Inf	1.3–Inf	0.02
<i>Ochlerotatus rusticus</i>	35	0	Inf	0.13–Inf	NS	Inf	0.9–Inf	NS	Inf	0.5–Inf	NS
<i>Aedes cinereus/geminus</i>	34	1	0.47	0.08–19.4	NS	3.5	0.6–140	NS	2	0.3–80	NS
<i>Culiseta annulata</i>	31	1	0.4	0.07–17.7	NS	3.1	0.5–128	NS	1.78	0.29–72.8	NS
<i>Culex pipiens</i>	23	32	0.01	0.006–0.02	< 0.0001	0.07	0.04–0.12	< 0.0001	0.04	0.02–0.07	< 0.0001
<i>Hulecoeteomyia japonica</i>	27	0	Inf	0.1–Inf	NS	Inf	0.7–Inf	NS	Inf	0.4–Inf	NS
<i>Ochlerotatus sticticus</i>	8	0	Inf	0.02–Inf	NS	Inf	0.2–Inf	NS	Inf	0.1–Inf	NS
<i>Dahlia geniculata</i>	2	0	Inf	0.003–Inf	NS	Inf	0.02–Inf	NS	Inf	0.01–Inf	NS
<i>Culiseta morsitans</i>	2	0	Inf	0.003–Inf	NS	Inf	0.02–Inf	NS	Inf	0.01–Inf	NS
<i>Culex territans</i>	1	0	Inf	0.0035–Inf	NS	Inf	0.003–Inf	NS	Inf	0.001–Inf	NS
<i>Ochlerotatus cataphylla</i>	1	0	Inf	0.0035–Inf	NS	Inf	0.003–Inf	NS	Inf	0.001–Inf	NS
Total§	1010	58	0.24	0.19–0.32	< 0.0001	1.8	1.4–2.3	< 0.0001	1.04	0.77–1.33	NS

\*Body weight: BW<sub>horse</sub> = 570 kg; BW<sub>chicken</sub> = 8 kg (four chickens of 2 kg each).

†Body surface area: (m<sup>2</sup>) = 0.11 × body weight (kg)<sup>0.65</sup>; BSA<sub>horse</sub> = 6.8 m<sup>2</sup>; BSA<sub>chicken</sub> = 0.11 × 2<sup>0.65</sup> × 4 = 0.69 m<sup>2</sup>.

‡Kleiber's scaling factor: I (kJ × kg) = I<sub>0</sub> × m<sup>0.75</sup>; I, metabolism; I<sub>0</sub>, normalization constant (= 283 kJ); mm body weight (kg); I<sub>horse</sub> = 33 014 kJ × kg; I<sub>chicken</sub> = I<sub>0</sub> × 2<sup>0.75</sup> × 4 = 1904 kJ × kg.

§Includes an additional six mosquitoes that were not identifiable (three *Aedes* collected in the horse cage; three *Culex/Coquillettidia* collected in the chicken cage).

95% CI, 95% confidence interval; Inf, infinity; NS, not significant.

chicken-baited traps or by human baiting. *Hulecoeteomyia japonica* was collected in the horse-baited trap as well as by human landings. Six of the 15 different mosquito species were collected in both the horse- and the chicken-baited traps (Table 1). The species *O. cantans/annulipes*, *Adm. vexans*, *Ae. cinereus/geminus*, *Cs. annulata* and *Cx. pipiens* were obtained from both animal species.

Data analyses revealed that *An. claviger* was significantly more attracted by the horse than by the chickens when findings were adjusted for BW ( $P=0.02$ ), BSA and KSF ( $P<0.0001$  for both) (Table 2). Evidence that *O. cantans/annulipes*, *Adm. vexans* and *An. maculipennis s.l.* showed a preference for the horse was apparent when the data were analysed according to BSA ( $P<0.0001$ ) or for KSF ( $P<0.001$ ) (Table 2). Chicken was significantly more attractive than horse in all three corrected values for *Cq. richiardii* (BW,  $P<0.0001$ ; BSA,  $P=0.02$ ; KSF,  $P<0.0001$ ) and *Cx. pipiens* ( $P<0.0001$  for all factors).

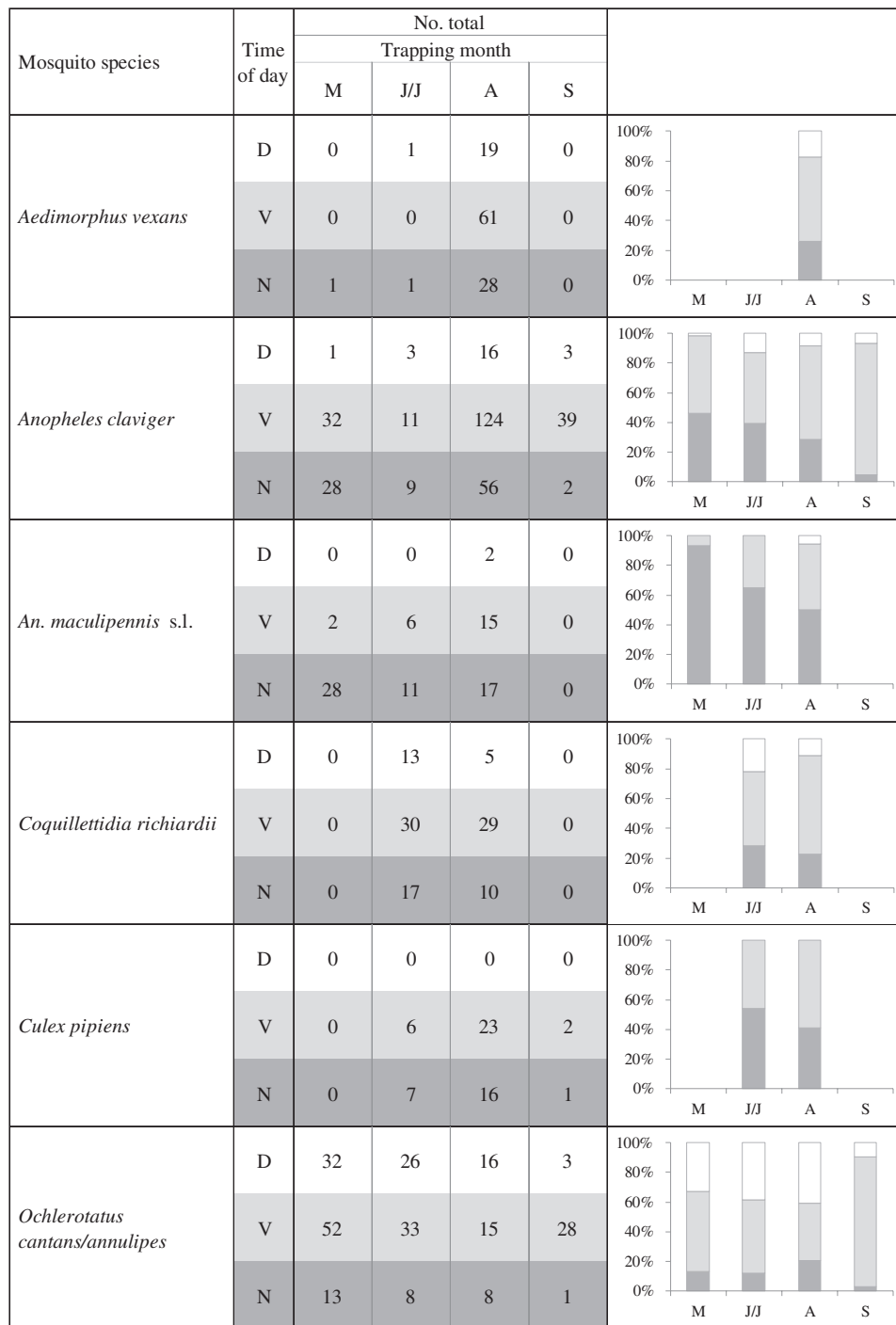
**Seasonal and diurnal activity.** The seasonal and diurnal activities of the six most abundant mosquito species are shown in Fig. 3. The two most frequently collected mosquito species, *An. claviger* and *O. cantans/annulipes*, were abundant over the whole trapping season from May to September, whereas the other species showed restricted seasonal occurrences. *Anopheles maculipennis s.l.* disappeared late in the season; *Adm. vexans*, *Cq. richiardii* and *Cx. pipiens* were prevalent during summer. *Ochlerotatus cantans/annulipes* had mainly diurnal and vespertine activity, whereas *An. claviger* was mainly active at

dusk and during the night (Fig. 3). The activity pattern of *An. maculipennis s.l.* was similar to that of *An. claviger*. *Coquillettidia richiardii* and *Adm. vexans* showed activity in all of the periods investigated, whereas *Cx. pipiens* was collected only during the evening and at night.

The activity patterns of the less abundant species (total numbers collected: 27–35 specimens) (Table 2) showed mainly diurnal and vespertine activity in *O. rusticus*, which was collected only at the first trapping in May. *Aedes cinereus/geminus* and *Cs. annulata* were mainly active at dusk and during the night, and were most prevalent during June–September in the former and in August in the latter case. *Hulecoeteomyia japonica* was collected during the whole season mainly at dusk.

#### Bloodmeal analysis of blood-fed mosquitoes

**Mosquito collections.** In total, 385 blood-fed mosquitoes belonging to nine different mosquito species of four genera were collected at Zoo Zürich (152 in 2013 and 233 in 2014). The five different traps employed in 2013 between June and August yielded only 13 (8.6%) blood-fed mosquitoes; the other 139 were collected by aspiration at 10 different sites. In 2014, only aspiration was performed at 21 different resting sites. Identification of blood hosts was successful in 92.5% of the mosquitoes with a bloodmeal of Sella stage II, 93.5% of those with bloodmeals of Sella stages III or IV and 86.3% of those with bloodmeals of Sella stages V and higher.



**Fig. 3.** Numbers of females of the six most abundant mosquito species collected in animal-baited traps at both sites during the four collection periods and at different times of day. In the graphs, only collections of at least 13 mosquitoes per collection date are considered. D, diurnal; N, nocturnal; V, vespertine; M, May; J/J, June/July; A, August; S, September.

At the field sites, a total of 181 (22 in 2012 and 159 in 2013) blood-fed females belonging to 12 different mosquito species from five genera were collected, of which the vast majority (92.8%) were collected in the area south of the Alps. A total of 165 females (91.2%) were trapped with

CO<sub>2</sub>-iGu CDC traps in 2013 and 135 of these were successfully analysed for their blood host. Further bloodmeal sources were identified from CO<sub>2</sub>-baited CDC traps without iGu (13 females) and from collections with backpack aspirators [two females, *Culiseta morsitans* (Theobald)]. Insects trapped with resting

traps, gravid traps, BGS and BGM traps or by netting did not yield additional data (as a result of the low numbers of trapped mosquitoes and failure of genetic analyses).

Of the 208 blood-fed *Cx. pipiens/torrentium* collected, 50 were identified to species level by PCR in pools which all tested positive for *Cx. pipiens* and negative for *Cx. torrentium*.

In total, *Cx. pipiens/torrentium* was the mosquito species most frequently collected (59.4% of all blood-fed mosquitoes), followed by *H. japonica* (16.7%) and *Adm. vexans* (12.4%) (Table 3).

*Mosquito blood hosts at Zoo Zürich.* In 354 (91.9%) of the blood-fed mosquitoes, bloodmeal sources were successfully identified through various PCR assays (Table 3). Bloodmeals were initially analysed with PCR primer pairs with pretended specificities for avians and mammals, respectively (Ngo & Kramer, 2003). However, sequencing of 114 amplicons obtained with the avian-specific primers yielded a mammalian host species in 20 and a reptile host in one case. Of the 108 samples initially analysed with mammalian-specific primers, 11 resulted in positive reactions and sequencing confirmed the mammalian origin. However, another 26 samples were negative with this primer pair, but the mammalian origin of the blood source was eventually proven by further analysis with other, vertebrate-specific, primers (Kocher *et al.*, 1989; Townzen *et al.*, 2008), which were used for analyses of all remaining samples.

In total, 35 different host species, including 17 birds, 17 mammals and one reptile, were identified in 338 bloodmeals (Table 3). A further 16 bloodmeals yielded identical sequences that could not be unambiguously attributed to a bird species (good quality sequences of 163–403 bp; best match in GenBank 93% with the bird species white spoonbill *Platalea leucorodia*, which is present at the study site). Amino acid translation of this sequence ([www.ebi.ac.uk/Tools/st/](http://www.ebi.ac.uk/Tools/st/)) revealed a coding region with 97% identify with this bird species.

No identification was obtained in a total of 31 (8.1%) specimens as a result of PCR failure, or poor quality or ambiguous spectra obtained upon direct sequencing of the amplicons. In six of these specimens, amplicons were cloned and four clones of each were sequenced. Thus, the blood hosts were determined in four cases, among which was one mixed bloodmeal (house sparrow, New World camelid) in an *H. japonica*. Two of the mosquitoes analysed also yielded mosquito sequences and the remaining two specimens yielded only mosquito sequences.

Exclusively avian hosts were identified in 233 (65.8%), mammals in 114 (32.2%), and reptiles in six (1.7%) of the samples. The major avian blood hosts identified in a total of 178 (76.4%) of the avian bloodmeals were Humboldt's penguin (*Spheniscus humboldti*,  $n = 70$ ), the house sparrow (*Passer domesticus*,  $n = 50$ ), the blackbird (*Turdus merula*,  $n = 41$ ) and the blue tit (*Cyanistes caeruleus*,  $n = 17$ ). Each of the other 13 bird species was identified in fewer than 10 specimens (Table 3). The most frequent mammalian host species were New World camelids (*Lama glama/guanicoe/pacos*,  $n = 56$ ) and the human (*Homo sapiens*,  $n = 18$ ), followed by the cat (*Felis catus/silvestris*,  $n = 8$ ), Asiatic elephant (*Elephas maximus*,  $n = 7$ ), donkey (*Equus asinus*,  $n = 7$ ) and sheep (*Ovis aries*,

$n = 5$ ). Each of the other 11 mammalian species was detected only once or twice (Table 3).

None of the four most frequently collected mosquito species took blood exclusively from one host class. *Culex pipiens/torrentium* had 197 bloodmeals originating from avian hosts and nine bloodmeals from mammalian hosts, thus demonstrating a significant preference for the former host type (Table 3). By contrast, *H. japonica* and *Adm. vexans* significantly more often ingested blood from mammalian hosts. *Anopheles maculipennis s.l.* showed no host preference.

*Mosquito blood hosts in field-collected mosquitoes.* In field-collected mosquitoes, a total of 151 bloodmeals (83.4%) representing 13 host species were identified (Table 3). Five bloodmeals (3.3%) derived from four different wild avian host species, including the magpie (*Pica pica*,  $n = 2$ ), blue tit (*Cyanistes caeruleus*,  $n = 1$ ), mallard (*Anas platyrhynchos*,  $n = 1$ ) and song thrush (*Turdus philomelos*,  $n = 1$ ). The rest of the bloodmeal sources were mammals and included five different livestock species (59.6%), three wild animal species (32.5%) and humans (4.6%) (Table 3). The most common mammalian livestock and wild species were domestic cattle (*Bos taurus*, 56.3%) and roe/red deer (*Capreolus capreolus*; *Cervus elaphus*, 32.4%). *Aedimorphus vexans*, *O. cantans/annulipes* and *Ochlerotatus sticticus* (= *Aedes sticticus*) (Meigen), the three most frequently collected species, had strong preferences for mammals, overwhelmingly cattle. Only a few specimens of each of the other nine species were collected.

## Discussion

Host preferences of mosquitoes were investigated in Switzerland by repeated animal bait experiments using a horse and four chickens at a natural and a suburban site, as well as by the analysis of blood-fed mosquitoes collected over 2 years at a zoo and at four field study sites. The purpose of the study was to contribute to a risk assessment for WNV transmission in Switzerland. The important characteristics that qualify a mosquito species to act as a bridge vector of WNV include opportunistic feeding behaviour on both avian and mammalian hosts and high abundance in late summer and autumn when human WNF cases generally occur (see weekly updates of human cases at <http://ecdc.europa.eu>). Overall, 17 of the 41 mosquito species recorded in Switzerland (Schaffner & Mathis, 2013) were collected in the present experiments, although five species [*Ochlerotatus cataphylla* (= *Aedes cataphylla*) (Dyar), *Dahlia geniculata* (= *Aedes geniculatus*) (Olivier), *Anopheles plumbeus* (Stephens), *Cs. morsitans* and *Culex territans* (Walker)] were very rare (five or fewer specimens obtained), which confirms earlier findings from a more extended survey in the study area (Schaffner & Mathis, 2013). These species are not discussed further. Of the European mosquito species that are implicated as WNV vectors (*Cx. pipiens*, *Cx. modestus*) (Hubálek, 2000; Medlock *et al.*, 2005), the latter, which is the main WNV vector in southern France (Balenghien *et al.*, 2006), was not detected in the present study. This species is rarely found in Switzerland and then only in the south of the country.

**Table 3.** Host species of blood-fed mosquitoes collected at Zoo Zürich and at other field sites in Switzerland

Mosquito species	Bloodmeals, <i>n</i> (95% CI)				Host species (Latin names; bloodmeals, <i>n</i> )	
	Mammal	Avian	Reptile	Mixed	Zoo Zürich	Field
<i>Culex pipiens/torrentium</i>	9 (4–18)	199* (172–229)	0 (0–4)	0 (0–4)	Mammal ( <i>n</i> = 9): human ( <i>Homo sapiens</i> ; 6), cat ( <i>Felis catus/silvestris</i> ; 3) Avian ( <i>n</i> = 197): Humboldt's penguin ( <i>Spheniscus humboldti</i> ; 65), house sparrow ( <i>Passer domesticus</i> ; 41), blackbird ( <i>Turdus merula</i> ; 35), blue tit ( <i>Cyanistes caeruleus</i> ; 13), unknown bird species (13), great tit ( <i>Parus major</i> ; 7), carrion crow ( <i>Corvus corone</i> ; 5), peacock ( <i>Pavo cristatus</i> ; 4), Egyptian vulture ( <i>Neophron percnopterus</i> ; 3), chicken ( <i>Gallus gallus</i> ; 2), Flightless steamerduck ( <i>Tachyeres pteneres</i> ; 2), pied wagtail ( <i>Motacilla alba</i> ; 2), collared dove ( <i>Streptopelia decaocto</i> ; 1), Demoiselle crane ( <i>Anthropoides virgo</i> ; 1), Patagonian conure ( <i>Cyanoliseus patagonus</i> ; 1), plush-crested jay ( <i>Cyanocorax chrysops</i> ; 1), sparrow hawk ( <i>Accipiter nisus</i> ; 1)	Avian ( <i>n</i> = 2): magpie ( <i>Pica pica</i> ; 1), mallard ( <i>Anas platyrhynchos</i> ; 1)
<i>Aedimorphus vexans</i>	137* (115–162)	9 (4–18)	0 (0–4)	0 (0–4)	Mammal ( <i>n</i> = 35): New World camelid ( <i>Lama glama/guanicoe/pacos</i> ; 10), human (7), Asiatic elephant ( <i>Elephas maximus</i> ; 6), donkey ( <i>Equus asinus</i> ; 6), cat (3), blackbuck ( <i>Antilope cervicapra</i> ; 1), horse ( <i>Equus caballus</i> ; 1), sheep ( <i>Ovis aries</i> ; 1) Avian ( <i>n</i> = 8): great tit (2), house sparrow (2), blackbird (1), blue tit (1), Egyptian vulture (1), unknown bird species (1)	Mammal ( <i>n</i> = 102): cattle ( <i>Bos taurus</i> ; 65), red deer ( <i>Cervus elaphus</i> ; 25), roe deer ( <i>Capreolus capreolus</i> ; 7), goat ( <i>Capra hircus</i> ; 1), horse (1), human (1), sheep (1), red fox ( <i>Vulpes vulpes</i> ; 1) Avian ( <i>n</i> = 1): magpie (1)
<i>Hulecoeteomyia japonica</i>	50* (37–66)	9 (4–18)	0 (0–4)	1 (0–6)	Mammal ( <i>n</i> = 48): New World camelid (39), human (2), sheep (2), dog ( <i>Canis lupus familiaris</i> ; 1), donkey (1), harbour seal ( <i>Phoca vitulina</i> ; 1), Indian lion ( <i>Panthera leo persica</i> ; 1), nilgai ( <i>Boselaphus tragocamelus</i> ; 1) Avian ( <i>n</i> = 9): chicken (3), Darwin's rhea ( <i>Pterocnemia pennata</i> ; 2), blackbird (1), house sparrow (1), Humboldt's penguin (1), unknown bird species (1) Mixed ( <i>n</i> = 1): house sparrow and New World camelid (1)	Mammal ( <i>n</i> = 2): human (2)
<i>Ochlerotatus cantans/annulipes</i>	19*(11–30)	0 (0–4)	0 (0–4)	0 (0–4)		Mammal ( <i>n</i> = 19): roe deer (12), cattle (4), human (2), dog (1)

**Table 3.** Continued

Mosquito species	Bloodmeals, <i>n</i> (95% CI)				Host species (Latin names; bloodmeals, <i>n</i> )	
	Mammal	Avian	Reptile	Mixed	Zoo Zürich	Field
<i>Anopheles maculipennis s.l.</i>	9 (4–18)	10 (4–19)	0 (0–4)	0 (0–4)	Mammal ( <i>n</i> = 7): New World camelid (3), human (1), sheep (1), South American tapir ( <i>Tapirus terrestris</i> ; 1), spectacled bear ( <i>Tremarctos ornatus</i> ; 1) Avian ( <i>n</i> = 10): blackbird (3), Humboldt's penguin (3), blue tit (2), house sparrow (1), unknown bird species (1)	Mammal ( <i>n</i> = 2): cattle (2)
<i>Ochlerotatus sticticus</i>	10*(4–19)	0 (0–4)	0 (0–4)	0 (0–4)		Mammal ( <i>n</i> = 10*): cattle (9), human (1)
<i>Culex hortensis</i>	3 (0–9)	1 (0–6)	5 (1–12)	0 (0–4)	Mammal ( <i>n</i> = 3): cat (2), human (1) Avian ( <i>n</i> = 1): blue tit (1) Reptile ( <i>n</i> = 5): common wall lizard ( <i>Podarcis muralis</i> ; 5)	
<i>Aedes</i> spp.	6*(2–14)	1 (0–6)	0 (0–4)	0 (0–4)	Mammal ( <i>n</i> = 3): Asiatic elephant (1), New World camelid (1), roe deer (1) Avian ( <i>n</i> = 1): Darwin's rhea (1)	Mammal ( <i>n</i> = 3): cattle (2), red deer (1)
<i>Coquillettidia richiardii</i>	5*(1–12)	0 (0–4)	0 (0–4)	0 (0–4)		Mammal ( <i>n</i> = 5*): cattle (2), roe deer (3)
<i>Culiseta annulata</i>	5*(1–12)	0 (0–4)	0 (0–4)	0 (0–4)	Mammal ( <i>n</i> = 5*): New World camelid (3), nilgai (1), roe deer (1)	
<i>Anopheles plumbeus</i>	2 (0–8)	2 (0–8)	0 (0–4)	0 (0–4)	Mammal ( <i>n</i> = 2): human (1), manul ( <i>Otocolobus manul</i> ; 1) Avian ( <i>n</i> = 2): blackbird (1), house sparrow (1)	
<i>Culex territans</i>	0 (0–4)	3 (0–9)	1 (0–6)	0 (0–4)	Avian ( <i>n</i> = 3): house sparrow (2), Humboldt's penguin (1) Reptile ( <i>n</i> = 1): common wall lizard (1)	
<i>Dahlia geniculata</i>	3 (0–9)	0 (0–4)	0 (0–4)	0 (0–4)	Mammal ( <i>n</i> = 2): cattle (1), sheep (1)	Mammal ( <i>n</i> = 1): cattle (1)
<i>Culiseta morsitans</i>	1 (0–6)	1 (0–6)	0 (0–4)	0 (0–4)		Mammal ( <i>n</i> = 1): human (1) Avian ( <i>n</i> = 1): song thrush ( <i>Turdus philomelos</i> ; 1)
<i>Culex</i> spp.	0 (0–4)	2 (0–8)	0 (0–4)	0 (0–4)	Avian ( <i>n</i> = 2): house sparrow (2)	
<i>Aedes cinereus/geminus</i>	0 (0–4)	1 (0–6)	0 (0–4)	0 (0–4)		Avian ( <i>n</i> = 1): blue tit (1)
<i>Anopheles claviger</i>	1 (0–6)	0 (0–4)	0 (0–4)	0 (0–4)		Mammal ( <i>n</i> = 1): roe deer (1)

\*Statistically significant (95% CI) preference for mammals or birds, respectively. 95% CI, 95% confidence interval.

No species was identified in both approaches (animal bait, blood host analyses) as being exclusively ornithophilic, whereas three species (*An. claviger*, *O. rusticus* and *O. sticticus*) fed only on mammalian blood or were found only in the horse trap (Tables 2 and 3). However, because relatively low numbers of the latter two species were retrieved from the horse-baited trap (*n* = 35 and *n* = 8, respectively), this was not statistically significant when data were corrected for features of the animal bait species (BW, BSA and KSF). None of these species has been implicated as a WNV vector (Hubálek & Halouzka, 1999; Medlock *et al.*, 2005; Becker *et al.*, 2010).

Nine species were identified as having varying degrees of opportunistic feeding behaviour with regard to avian and mammalian hosts; this blood-feeding plasticity is widespread among

mosquito species (Chaves *et al.*, 2010; Takken & Verhulst, 2013). *Culex pipiens*, the major WNV vector in European endemic areas (Campbell *et al.*, 2001; Esteves *et al.*, 2005; Garcia-Bocanegra *et al.*, 2012; Mulatti *et al.*, 2014), showed a strong preference for birds that was statistically highly significant in the animal bait experiments for all three corrected factors (Table 2) and also for the analysed blood-fed specimens (Table 3). This is similar to findings in previous studies (Molaei *et al.*, 2006; Tuten *et al.*, 2012; Osorio *et al.*, 2014). The species was attracted to horses and, interestingly, preferred humans among the mammalian hosts identified in specimens collected at the zoo (Table 3), as has been described previously (Tuten *et al.*, 2012; Osorio *et al.*, 2014). No attempts were made to distinguish *Cx. pipiens pipiens* and its strongly anthropophilic

biotype *molestus*, which prevails in urban settings (Medlock *et al.*, 2005) and which may also have been present at the zoo site. Nevertheless, *Cx. pipiens* was also attracted to mammalian hosts at rural sites, from which the biotype *molestus* may be absent. Undoubtedly, *Cx. pipiens*, which is widespread in Switzerland (Schaffner & Mathis, 2013) and which is highly abundant in summer and autumn (Fig. 3), would be a key vector for the transmission of WNV if the virus were to be introduced into Switzerland. Analyses by PCR of a large proportion of the *Cx. pipiens/torrentium* specimens collected showed no indication of the presence of *Cx. torrentium*, which differs in its vector competence traits and which prevails in more northerly European regions (Hesson *et al.*, 2014) and at higher altitudes in central Europe (F. Schaffner, unpublished data, 2013).

*Hulecoeteomyia japonica*, which is native to northeastern Asia, is an invasive mosquito species in the U.S.A. and more recently in Europe, where there are currently six stable populations discontinuously distributed over temperate central Europe, which show a tendency to spread further (Schaffner *et al.*, 2009; Kampen & Werner, 2014). The species can rapidly become highly abundant in newly colonized territories (Schaffner *et al.*, 2009; Anderson *et al.*, 2012; Kampen & Werner, 2014) as a result of the ecological plasticity of its larvae, which presumably can out-compete larvae of other species (Kampen & Werner, 2014). *Hulecoeteomyia japonica* prefers forested and bushy habitats in rural, suburban and urban environments (Andreadis *et al.*, 2001; Bartlett-Healy *et al.*, 2012), but was also present at the natural site in the current study (Table 1). Its known host preference for mammals (Scott, 2003; Apperson *et al.*, 2004; Molaei *et al.*, 2009) was confirmed in the present study, in which the species was also shown for the first time to feed to a considerable extent on birds in the field (17.2% of *H. japonica* collected in the zoo had fed on avians) (Table 3). Interestingly, the only mixed bloodmeal identified in the present study was from an *H. japonica* collected in the zoo that had fed on both a bird and a mammal. This mosquito species has been considered as a possible bridge vector for WNV based on the identification of virus-carrying specimens collected in the field in the U.S.A. and several respective laboratory vector competence studies (Schaffner *et al.*, 2013), although results from corresponding experiments with European populations were controversial (Huber *et al.*, 2014; S. Wagner *et al.*, unpublished data, 2015). Given that the species is abundant throughout the WNV transmission season, it should be considered as a main candidate bridge vector.

As in other studies (Balenghien *et al.*, 2006; Greenberg *et al.*, 2011), *Adm. vexans* showed a significant host preference for mammals, although this was statistically significant in the animal bait experiments only with regard to BSA ( $P=0.003$ ) (Table 2). In addition, 18.6% of the blood-fed *Adm. vexans* collected at the zoo had fed on birds. The species was found to be a moderate vector for WNV under laboratory conditions (Turell *et al.*, 2005; Tiawsirisup *et al.*, 2008). Extremely high abundances of this species can be observed depending on the timing of local floodings and may possibly occur during the WNV transmission season. Indeed, a relatively short population peak in summer (August) (Fig. 3) was observed in the present study. Thus, a putative role of *Adm. vexans* as a bridge vector

of WNV has to be considered in areas with expanded suitable breeding habitats.

*Coquillettidia richiardii*, another possible bridge vector of WNV in Europe (Hubálek & Halouzka, 1999; Medlock *et al.*, 2005), also showed a distinct preference for avians in the host bait experiments (Table 2). Nevertheless, a considerable number were attracted to the horse bait and all of the few blood-fed specimens ( $n=5$ ) collected at the field sites were found to have fed on mammals (Table 3). The species, which depends on a particular breeding site with erect aquatic plants in permanent waters, was the fourth most abundant at the natural site but was very rare at the periurban site, as is the case in most parts of Switzerland (Schaffner & Mathis, 2013). Given that it peaks in abundance before the high-risk season for zoonotic WNV transmission in late summer, this species may be locally involved, particularly in enzootic transmission.

The remaining species identified in the present study as having opportunistic feeding behaviour [*O. cantans/annulipes*, *Ae. cinereus/geminus*, *An. maculipennis s.l.*, *Cs. annulata* and *Culex hortensis* (Ficalbi)] seem to be of minor importance with regard to bridge vector function. None of them has been implicated in WNV transmission in the field, although viral DNA has been identified in *O. cantans/annulipes* and *An. maculipennis s.l.* collected in the field (Hubálek & Halouzka, 1999; Kemenesi *et al.*, 2014). *Ochlerotatus cantans/annulipes*, the second most abundant species retrieved in the animal bait experiments, which was active from spring to autumn, as well as *Ae. cinereus/geminus* and *Cs. annulata*, almost exclusively preferred mammalian hosts (Tables 2 and 3). This holds true for *An. maculipennis s.l.* collected in the animal bait experiments, but, surprisingly, half of the bloodmeals identified in specimens collected at the zoo were of avian origin. It remains to be determined whether this may reflect the presence of different species of the *Maculipennis* complex. *Anopheles maculipennis s.s.* and *Anopheles messeae* (Falleroni) are known to occur in Switzerland (Schaffner & Mathis, 2013), and the latter has been shown to bite both birds and mammals (Danabalan *et al.*, 2014). *Culex hortensis*, collected only at the zoo, was a truly catholic feeder (reptiles, mammals, birds), although bloodmeals in only a few specimens could be analysed.

As expected, species diversity was higher at the natural site than at the periurban site in the animal bait experiments. Five of the six most abundant mosquito species were present at the natural as well as the periurban site (*An. maculipennis s.l.* was present only at the natural site). Mosquito numbers collected per 12 h in the animal bait experiments were on average five times higher at the natural site than at the periurban site. Differences between the study sites resulted in mosquito numbers that were twice (May), five times (June/July) and 46 times (August) higher at the natural site, although collection numbers in September were equal. The windy weather conditions during the last animal bait experiment at the natural site may explain the latter result. In comparison with a similar study performed in southern France (Balenghien *et al.*, 2006), 14 times fewer mosquitoes were collected per 12 h in wet areas in the present study (respectively: the Camargue region and the natural site), but similar mosquito numbers were collected in the dry and periurban areas. Lower mosquito densities in Switzerland may indicate a lower risk for transmission of WNV.



Humans seem to represent an attractive host for mosquito fauna. Overall, 9.6% of the bloodmeals of mosquitoes collected at Zoo Zürich originated from humans, which thus represented the second most frequent mammalian host after New World camelids. This is in agreement with the findings of an earlier study from the U.S.A. (Tuten *et al.*, 2012). Interestingly, all of the putative WNV bridge vectors discussed herein (*Cx. pipiens*, *H. japonica*, *Adm. vexans*, *Cq. richiardii*) were attracted to humans (Tables 1 and 3).

Analyses of the blood-fed mosquitoes from the zoo and the field with different primer pairs resulted in the successful identification of blood hosts in 91.9% of specimens, which is higher than in previous studies (Townzen *et al.*, 2008; Lassen *et al.*, 2012; Tuten *et al.*, 2012; Mehus & Vaughan, 2013) in which single or multiple (as in the present study) PCR approaches were employed. The primers used in the present study (Kocher *et al.*, 1989; Ngo & Kramer, 2003; Townzen *et al.*, 2008) were not sensitive (i.e. 'mammalian primers' did not detect all mammalian hosts, such as New World camelids, which required an approach using 'universal vertebrate primers') or did not have the declared specificities [i.e. they also amplified pseudogenes (as became obvious in the analyses of the sequences translated; data not shown) or mosquito sequences (as became obvious after the sequencing of cloned amplicons)]. Thus, the assays chosen are far from being optimal and primers that perform better are urgently needed.

## Conclusions

The two approaches to determining the host preferences (mammal vs. avian) of mosquitoes (animal-baited traps and analyses of the blood hosts of field-collected mosquitoes) yielded congruent results although with different levels of statistical significance. The former approach yielded more analysable species (i.e. reasonable numbers of specimens were collected), but is more elaborate. Based on the present results, and considering data on abundance, spatiotemporal activity, laboratory vector competence and virus detections in the field, *Cx. pipiens*, *H. japonica* and *Adm. vexans* are suggested to represent the candidates most likely to act as bridge vectors for the transmission of WNV in Switzerland.

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