Blood meal analysis, flavivirus screening, and influence of meteorological variables on the dynamics of potential mosquito vectors of West Nile virus in northern Italy

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ABSTRACT: An extended area of northern Italy has experienced several West Nile virus (WNV) outbreaks and the emergence of Usutu virus (USUV) during previous years. Our aim was to study some of the factors that could explain disease patterns in the Trentino region, where circulation was detected in human sera and sentinel chickens, but no human or equine cases were reported. We collected *Culex* species (Diptera: Culicidae) in peridomestic environments. The collected specimens were analyzed for feeding behavior, the influence of temperature and rainfall on the abundance of mosquitoes, and the occurrence of flaviruses. Analysis of blood meals showed that *Culex pipiens* fed mainly on blackbirds (*Turdus merula*) and house sparrows (*Passer domesticus*), while *Culex hortensis* fed strictly on lizards. The abundance of *Cx. pipiens* females correlated positively with mean temperature and negatively with rainfall (one to four weeks before capture). This negative relationship could be due to the direct effect of the flushing of habitats together with an indirect effect of oviposition repellency. The mean weekly temperature influenced the abundance of *Cx. hortensis*. No flaviviruses were detected in the analyzed *Culex* mosquitoes. These data suggest a silent cycle at low enzootic transmission levels in the area. Furthermore, we present the first contribution to understanding the transmission role of *Cx. pipiens* mosquitoes in Italy by identifying vertebrate hosts to species level. ***Journal of Vector Ecology* 37 (1): 20-28. 2012.**

Keyword Index: Culex, ecology, mosquitoes, West Nile virus, blood meal, climate.

INTRODUCTION

West Nile virus (WNV) (Flavivirus: Flaviviridae) is the world's most widespread arbovirus and occurs in all continents except Antarctica (Kramer et al. 2008). First isolated in Uganda in 1937 (Smithburn et al. 1940), it has caused several outbreaks of neuroinvasive disease and has emerged in new areas while re-emerging in others (Hayes and Gubler 2006). Following the European epidemic in Bucharest, Romania, during 1996-1997 (Tsai et al. 1998), the virus has emerged in several areas of the Mediterranean Basin and Europe (Czech Republic, Russia, Hungary, Greece, Spain) and re-emerged (Israel, France) with recent outbreaks of disease in northern Italy (Figure 1), with both human and equine neurological cases (Angelini et al. 2010).

WNV is an ecological generalist (multi-vector, multi-host) and has been detected in several mosquito and bird species in Europe (Hubálek and Halouzka 1999). It is generally accepted that WNV is maintained in an enzootic cycle involving ornithophilic *Culex* mosquito vectors and host birds (Kramer et al. 2008). In many locations, disease outbreaks are exacerbated by epizootic (bridge) vectors that transmit the virus from birds to horses or humans (dead-end hosts). Several *Culex* species are recognized as WNV vectors in Europe, such as *Culex modestus* in Camargue, France (Balenghien et al. 2006). The most important enzootic and bridge vector is *Cx. pipiens* in its two forms of *Cx. pipiens pipiens* (rural, feeds on birds) and *Cx. pipiens molestus* (urban, feeds on humans) (Fonseca et al. 2004). It has been suggested that *Cx. pipiens* was the epizootic and enzootic vector in Romania (Savage et al. 1999) and the epizootic vector in the Czech Republic (Hubálek 2000), Italy (Romi et al. 2004), and North America (Komar 2003). The feeding behavior of several *Culex* species is therefore crucial in affecting WNV epidemiology, but there are considerable differences between localities (Kilpatrick et al. 2006). However, there is a lack of information about feeding preferences of *Culex* mosquitoes in Europe, apart from other studies with less specific ELISA methods (Balenghien et al. 2006) and a recent study in Barcelona, Spain (Muñoz et al. 2011). The new PCR-based methodologies such as DNA barcoding improve the analysis to the vertebrate species level (Alcaide et al. 2009) and therefore may greatly contribute to the understanding of vector feeding behavior (GÓmez-Diaz and Figuerola 2010). The seasonal dynamics of the vector as it is affected by local climatic conditions are essential for allowing virus transmission (Ruiz et al. 2010). Temperature is an important parameter affecting mosquito abundance, modulating larval and pupal development in *Cx. pipiens* (Madder et al. 1983), increasing viral infection,

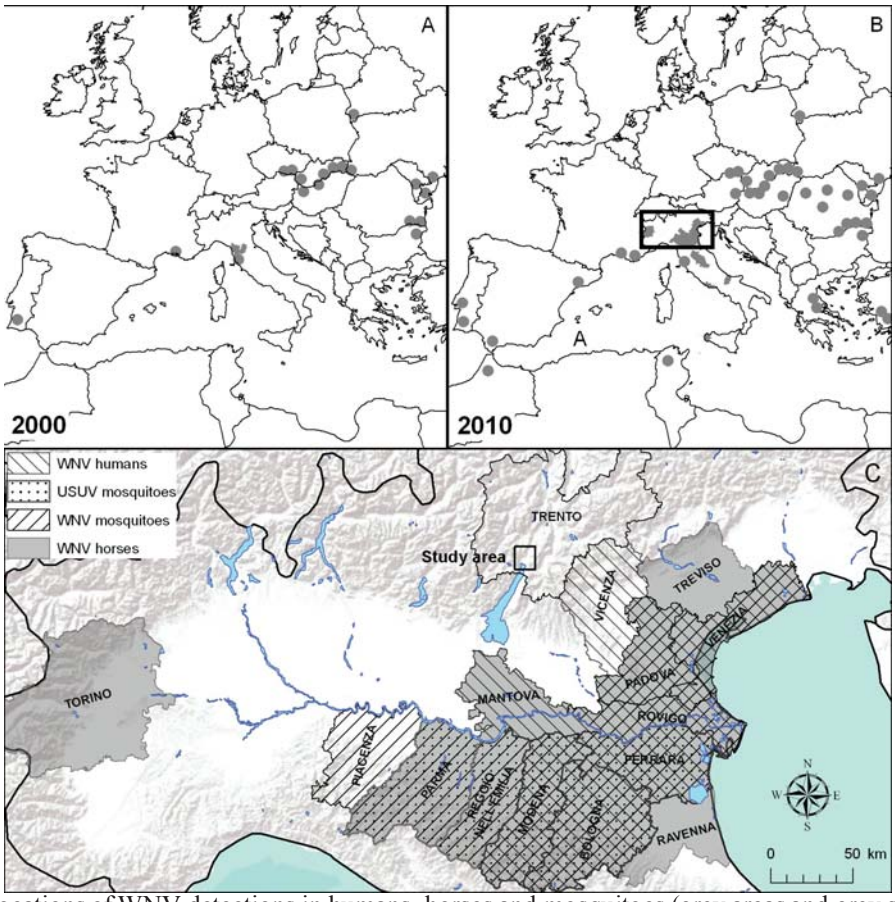

Figure 1. Geographic locations of WNV detections in humans, horses and mosquitoes (grey areas and grey spots) until 2000 (A) and until 2011 (B) in Europe and North Africa. Panel C shows the study area and provinces with human and horse cases and WNV and USUV mosquito positive pools during 2008-2011. Based on information reported by Hubalek and Halouzka 1999, Calistri et al. 2010, Calzolari et al. 2011, Angelini et al. 2010, Barzon et al. 2009, Rizzo et al. 2009, Macini et al. 2008, Monaco et al. 2009.

dissemination, and transmission of WNV (Kilpatrick et al. 2008), and determining mosquito population dynamics (Ruiz et al. 2010).

WNV and Usutu (USUV) viruses were detected in the province of Trento during the summer of 2005 with a high percentage (90%) of seroconversion in chickens, results that were confirmed by seroneutralization that implies a circulation of both viruses (Rizzoli et al. 2007). During 2002, 482 serum samples were collected from forestry workers and tested for antibodies to WNV by ELISA and neutralization tests. Thirty of 482 samples were positive, resulting in a mean prevalence of 6.1% (±1.1) (A. Rizzoli and W. Versini, unpublished data). From 2008 to 2010, several outbreaks of WNV in northern Italy and USUV were reported (Figure 1), suggesting *Cx. pipiens* was the principal vector (Angelini et al. 2010, Calzolari et al. 2011, Lelli 2010). New foci were observed in several other areas of Italy, confirming the ability of WNV to spread to new areas of Italy (Calistri et al. 2010).

Given the above scenario, our aim was to analyze some of the ecological parameters of the *Culex sp.* in this area. Our three research questions were: 1) Which are the hosts on which *Culex* mosquitoes are feeding? 2) How are temperature and rainfall related to *Culex* dynamics? 3) What are the flaviviruses present in *Culex* mosquitoes in Trentino?

MATERIALS AND METHODS

Study area

This study was conducted in the municipalities of Arco and Riva del Garda in in the province of Trento (Trentino-Alto Adige, Italy), an area with positive serologies in birds and humans and a warm climate. The municipalities of Arco (45° 55' N, 10° 53' E) and Riva del Garda (45° 53' N, 10° 50' E) have a sub-Mediterranean climate owing to their proximity to Lake Garda. The human population density of Riva del Garda and Arco in 2008 was 372.53 and 258.71 inhabitants per km^2, respectively.

Mosquito collections

From March to November, 2008 (weeks 14-48), adult mosquitoes were captured using BG-traps with the attractant BG-lure (BioGents, Germany) without CO_2. Twenty collection sites (ten in Arco and ten in Riva del Garda) were outdoor locations in suburban and

peridomestic habitats: school playgrounds, back gardens, and garden centers where trap continuity was guaranteed (density of one trap/0.5 km^2). The trap sites were recorded with a GPS. The mosquitoes were collected every 48 h and transported to the laboratory and stored at -20° C. Mosquito abundance data were aggregated by week.

Mosquito identification and storage

Frozen individuals were placed on white filter paper in a Petri dish on a chill table under a stereomicroscope and identified to species using the taxonomic keys of Schaffner et al. (2001). From one to 56 individuals were stored in 1.5 ml Eppendorf tubes containing 700 µl (>30 individuals) or 500 µl (<30 individuals) of MEM solution (Minimum Essential Medium Eagle, Invitrogen), separated by species, sex, locality, and date. Mosquito pools were crushed, homogenated using sterile and RNAase-free pestles, and then cold centrifuged. Supernatant (140 µl) was collected, transferred to a new tube and dissolved in 560 µl of AVL carrier/buffer RNA solution (Qiagen), and stored at -80° C. This lysis buffer preserved the RNA and rendered samples non-infectious (Blow et al. 2004). Blood-fed females were stored individually at -80º C while awaiting molecular blood meal identification.

Molecular identification of blood meal origin

Mosquito abdomens were separated from the rest of the bodies and placed into individual PCR tubes. DNA was isolated from the abdominal contents using the HotSHOT protocol (Truett et al. 2000) as described (Alcaide et al. 2009). DNA extracts from the mosquito blood meals served as the DNA template in a standard polymerase chain reaction (PCR) assay. PCR products were subsequently used for a nested PCR to amplify a fragment of a vertebrate cytochrome *c* oxidase subunit I (COI) mitochondrial gene using previously described primers (M13BC-FW/BCV-RV1 and M13-FW/BCV-RV2) and thermal cycling conditions (Alcaide et al. 2009). PCR reactions were carried out using a PTC-100 Programmable Thermal Controller (MJ Research). PCR amplified products were cleaned up using ExoSAP-IT (GE Healthcare Life Sciences). Sequencing reactions were performed using BigDye 1.1 technology (Applied Biosystems) with BCV-RV2 primer. Labeled DNA fragments were analyzed with an ABI 3130xl automated sequencer (Applied Biosystems). Sequences were checked using a Sequencher™ v.4.5 (Gene Codes Corp., ©1991–2005) and COI sequences were assigned to particular species by comparing them with the GenBank DNA sequence database (National Center for Biotechnology Information, 2008) and the BOLD Systems platform (http://www.boldsystems.org/views/login.php). Positive identification and host species assignment were based on exact or nearly exact matches (>98%).

Weather data

Daily mean, minimum, and maximum air temperatures and precipitation for the study period were obtained from the Arco meteorological station (http://meteo.iasma.it/meteo/). Data were pooled by week and mean weekly temperatures were computed. Total weekly precipitation was used for the rainfall dataset. In addition, a series of accumulated average temperatures and accumulated rainfall totals were calculated over the one to two, one to three, and one to four-week periods prior to the sampling week.

Statistical analysis

To explore the association between *Cx. pipiens* and *Cx. hortensis* abundance and climatic covariates, we performed linear regression models using the R statistical package version 2.13.1 (The R Foundation for Statistical Computing, 2011). To remove temporal autocorrelation within *Cx. pipiens* and *Cx. hortensis* adult female abundance, an autoregressive model selection procedure based on Akaike's Information Criterion (AIC) was implemented to select the best fitting correlation structure for seasonal mosquito abundance. The response variables considered for the climatic covariates models were the residual obtained from the best autoregressive models for mosquito abundance. As climatic covariates, we considered average weekly temperatures (minimum, maximum, mean), all accumulated temperature variables (see climate data section for details), total weekly precipitation, and all accumulated precipitation variables (see climate data section for details). All covariates were tested for multicollinearity, using Pearson product-moment correlation coefficients. The minimal models were chosen by AIC following a backward stepwise deletion procedure from the maximal initial model (Crawley 2002). Finally, to account for spatial correlation among the different traps, a mixed model with trap identification code as random factors was also implemented.

Virus identification by RT-PCR

Viral RNA was extracted from mosquitoes with the RNEasy miniKit (Qiagen extraction Kit (Qiagen)). Flavivirus RT-PCR following the Sánchez-Seco et al. (Sánchez-Seco et al. 2005) protocol with a modification in the nested PCR, where specific HotMaster Polymerase enzyme (Promega) was used to avoid non-specific amplicons.

RESULTS

Abundance and dynamics of mosquitoes

A total of 5,063 adult mosquitoes was trapped during the study period, including 2,639 females and 783 males of *Cx. pipiens* and 588 females and 1,053 males of *Cx. hortensis*. More *Cx. pipiens* females than males (77% and 23%, respectively) were captured with the BG-traps. In contrast, more males than females were detected for *Cx. hortensis* (64% and 36%, respectively). Dynamics of both *Culex* species showed a similar pattern, although *Cx. hortensis* populations increased quickly in the spring (Figure 2). Seventy-nine *Cx. pipiens* fed females and 12 *Cx. hortensis* fed females were stored for further analysis.

Blood meal analysis

Vertebrate DNA was successfully amplified from 45

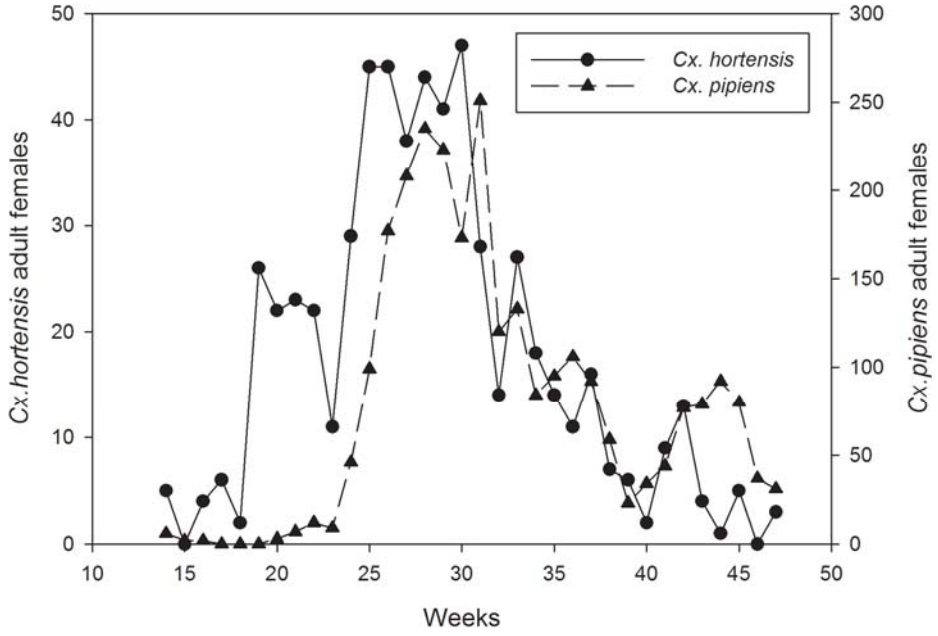

Figure 2. Number of female *Cx. pipiens* and *Cx. hortensis* trapped in each week interval between March and November 2008.

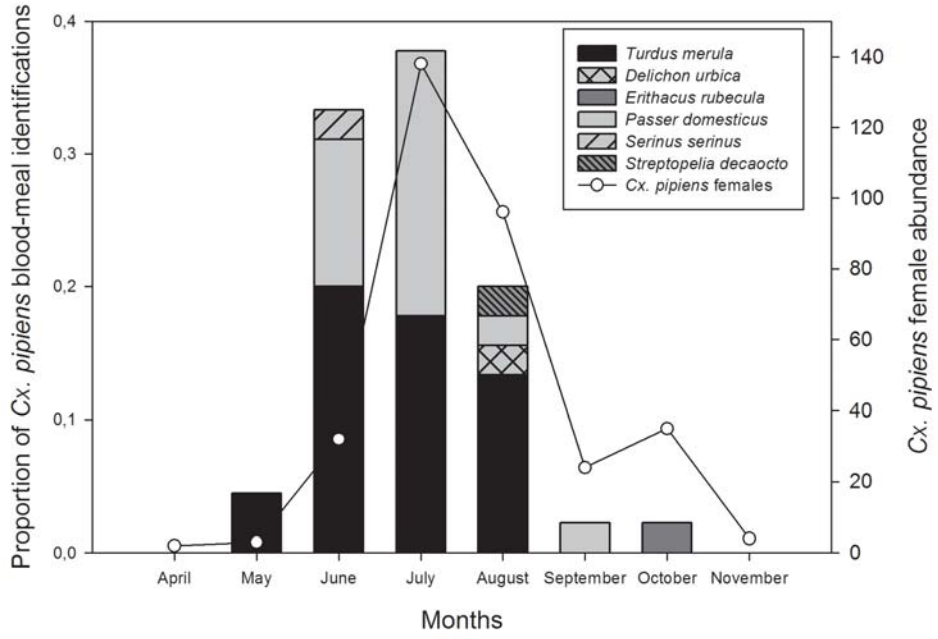

Figure 3. Origin of the blood meals of *Cx. pipiens*.

Cx. pipiens and eight *Cx. hortensis* blood-fed females. *Cx. pipiens* fed on *Turdus merula* L. (Common Blackbird; n=25), *Passer domesticus* L. (House Sparrow; n=16), *Delichon urbica* L. (House Martin; n=1), *Erithacus rubecula* (European Robin; n=1), *Serinus serinus* L. (European Serin; n=1), and *Streptopelia decaocto* Frivaldszky (Collared Dove; n=1). All *Cx. hortensis* blood meals came from the Common Wall Lizard (*Podarcis muralis* Laurenti). Figure 3 shows the seasonal pattern of blood meal origin in relation to *Cx. pipiens* female abundance. The most important host species for *Cx. pipiens* were *Turdus merula* and *Passer domesticus*, on which mosquitoes fed from May to August and from June to September, respectively.

Effect of temperature and rainfall on the dynamics of *Culex* species

Multicollinearity analysis indicated that the independent explanatory variables to be used in maximal initial models, both for *Cx. pipiens* and *Cx. hortensis*, were the average weekly mean temperature, the accumulated temperatures of one to four weeks prior to collection, the total week precipitation, and the accumulated rainfall of one to four weeks prior to collection.

The minimal adequate model explaining variation in the abundance of host-seeking adult *Cx. pipiens* females included the average weekly mean temperature and the rainfall of one to four weeks prior to collection (Table 1). Specifically, the average weekly mean temperature was positively related to *Cx. pipiens* female abundance (T-value=4.199; $p<0.001$), while the accumulated rainfall of one to four weeks prior to capture had a negative effect (T-value=-2.248; $p<0.05$) (Table 1, Figures 4 and 5). The minimal adequate model explaining variation in the abundance of host-seeking adult *Cx. hortensis* females included only the average mean weekly temperature, showing a positive relationship with the *Cx. hortensis* abundance (T-value=5.168; $p<0.001$) (Table 1, Figure 6). Adding trap identification code as a random factor did not improve the explained variance in any of these models.

Flavivirus screening

A total of 1,446 females (39 pools) of *Cx. pipiens* and 544 females (23 pools) of *Cx. hortensis* was analyzed. No flavivirus was detected in any of the pools.

DISCUSSION

The results presented from the blood meal analysis are of particular importance, being the first analysis of host feeding preferences for these vectors in Italy and one of the few in Europe that identifies hosts at the species level through DNA barcoding. This methodology helps to

Table 1. Results of the climatic covariate models for residuals of *Cx. pipiens* and *Cx. hortensis* female abundance**.**

Response variable	Explanatory variables	Coefficient (±S.E.)	t-value	P(>|t|)
Cx. pipiens female abundance residuals	Intercept	-0.5579 ± 0.2052	-2.719	<0.01
	Average weekly mean temperature	0.0433 ± 0.0103	4.199	<0.001
	Accumulated temperatures 1 to 4 weeks prior to collection	-0.0003 ± 0.0005	-0.598	0.55
	Total week precipitation	0.0018 ± 0.0012	1.489	0.14
	Accumulated rainfall 1 to 4 weeks prior to collection	-0.0023 ± 0.0009	-2.448	<0.05
Cx. hortensis female abundance residuals	Intercept	-0.6287 ± 0.1459	-4.307	<0.001
	Average weekly mean temperature	0.0381 ± 0.0074	5.168	<0.001
	Accumulated temperatures 1 to 4 weeks prior to collection	-0.0005 ± 0.0003	-1.504	0.13
	Total week precipitation	-0.0007 ± 0.0008	-0.865	0.39
	Accumulated rainfall 1 to 4 weeks prior to collection	0.0006 ± 0.0007	0.907	0.37

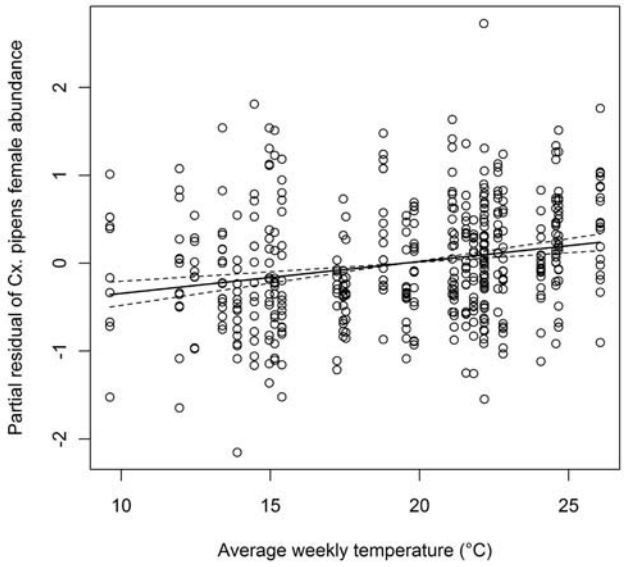

Figure 4. Relationship between average weekly temperature and *Cx. pipiens* female abundance residuals, after controlling for the effects of all other covariates in the model shown in Table 1. The solid line indicates the partial regression fit while dashed lines indicate standard error curves.

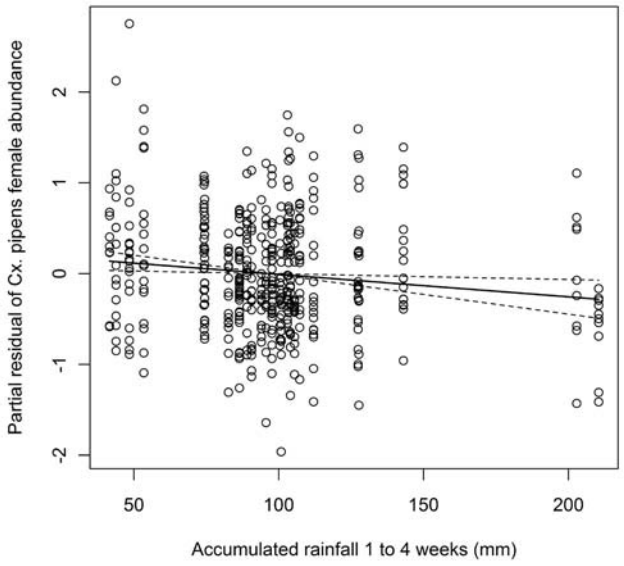

Figure 5. Relationship between accumulated rainfall 1 to 4 week and *Cx. pipiens* female abundance residual, after controlling for the effects of all other covariates in the model. The solid line indicates the partial regression fit while dashed lines indicate standard error curves.

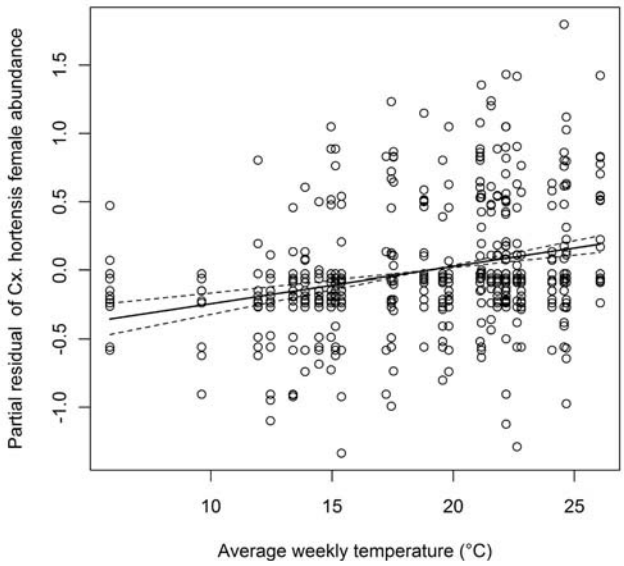

Figure 6. Relationship between average weekly temperature and *Cx. hortensis* female abundance residual, after controlling for the effects of all other covariates in the model. The solid line indicates the partial regression fit while dashed lines indicate standard error curves.

understand the host-*Culex* transmission network and allows the modeling of vertebrate mosquito contact rates (Alcaide et al. 2009, Gómez-Diaz and Figuerola 2010). In the study area, *Culex pipiens* was found to be an ornithophilic species, with blackbirds (*Turdus merula*) and house sparrows (*Passer domesticus*) as their principal hosts during the mosquito season despite the presence of numerous humans, especially during the summer when large numbers of tourists come to Garda Lake (more than 100,000 - Provincia Autonoma di Trento 2011). *Culex pipiens* was also identified as the principal vector in the recent WNV outbreaks in northern Italy (Angelini et al. 2010), but no studies on host vector preferences have been carried out to date. The marked ornithophilic behavior detected in our study suggests that the mosquito population in the province of Trento could belong to the *Cx. pipiens pipiens* ecotype (Fonseca et al. 2004), but further behavioral and genetic studies must be developed to confirm this. Despite the detection of WNV in this region, the ornithophilic preferences of *Cx. pipiens* could explain the absence of human or equine cases in 2005 and the absence of epizootic/epidemic cycles in the Trentino-Alto Adige region. In the U.S.A., the American robin (*Turdus migratorius*) has been suggested as a key species (super-spreader) in the diffusion of WNV (Kilpatrick et al. 2006). This work identifies *Turdus merula* and *Passer domesticus* as an important portion of the *Cx. pipiens* diet in this area. Unfortunately, we do not have data on the relative abundance of these host species during the same time frame. In addition, blood meal analyses confirmed that *Cx. hortensis* does not feed on mammals or birds in our study area feeding on the common wall lizard (*Podarcis muralis*), although previous authors have reported that this species is ornithophilic and herpetophilic. More studies are necessary to confirm the absence of an epidemiological role of *Cx. hortensis* in arbovirus transmission to mammals or birds in rural areas. Due to the lack of dry ice, we used BG traps with BG-lure attractants that were designed to collect anthropophilic mosquitoes but that also perform well for capturing ornithophilic mosquitoes such as *Cx. pipiens* in our study, as other studies have suggested (Molnar et al. 2006, Meeraus et al. 2008, Obenauer et al. 2009, Roiz et al. 2011), and herpetophilic mosquitoes such as *Cx. hortensis.*

The results show similar population dynamics for *Cx. pipiens* and *Cx. hortensis,* with *Cx. hortensis* more of an "early season" mosquito. *Culex* abundance is relatively low, and in general, this presents low WNV risks (Kramer et al. 2008). The statistical analysis of climate variables vs female abundance suggest that mean weekly temperatures and rainfall over the four weeks prior to capture affect the dynamics of *Cx. pipiens* in the Trentino region, with mean temperature having a positive effect and accumulated rainfall a negative effect. This negative effect of rainfall should be limited to specific climatic zones as the study area had frequent periods of high precipitation that had negative impacts due to the flushing of catch basins and containers that are important urban larval habitats (Koenraadt and Harrington 2008). Rains may also affect the fitness of the container for breeding mosquito larvae and pupae, diluting the nutrients for larvae (Dieng et al. 2010). There are also previous suggestions that there is an indirect effect through oviposition repellency. Since overflow has direct detrimental effects on mosquito larvae through splashing out, *Aedes albopictus* may avoid fully filled containers for oviposition and prefer to lay eggs in partly filled containers (Dieng et al. 2010). Although *Cx. pipiens* populations were more affected by excessive rain than *Aedes aegypti* (Koenraadt and Harrington 2008), it remains unknown if *Culex* species could experience oviposition repellency. The dynamics of *Cx. hortensis* were better explained by the mean weekly temperature and not by rainfall. The fact that *Cx. hortensis* uses permanent water sources and *Cx. pipiens* temporary ones as breeding sites (Gillet and Gilot 1983) could explain why *Cx. pipiens* was more dependent on rainfall. This study confirms the importance (already highlighted in other studies in other areas) of temperature and rainfall on the population dynamics of *Cx. pipiens* and other *Culex* mosquitoes (Ruiz et al. 2010). Temperature is a key factor for *Culex* dynamics and West Nile replication, therefore, insights into the influence of temperature in WNV vectors and virus replication in European lineages are important for evaluating the risk of WNV transmission and its spread into new areas of the continent.

Although seropositivity for WNV and USUV in sentinel chickens and humans was described in the Trentino-Alto Adige region in 2005 (Rizzoli et al. 2007), we did not detect WNV, USUV, or other flaviviruses in the *Culex* mosquitoes captured in 2008 in periurban areas. Nevertheless, this is not indicative of a total absence of WNV or USUV circulation in the area, but rather it is consistent with an enzootic cycle with low intensity of circulation, with *Cx. pipiens* as the principal vector. Surprisingly, we did not detect *Culex* flavivirus (Cx FV), a common insect flavivirus in other parts of the world (Kent et al. 2010), in agreement with results for neighboring regions, such as Lombardy (Calzolari et al. 2011).

The causes of the lack of a widespread spatial establishment but repeated emergence of West Nile and Usutu in Italy is an important research question. Interestingly, the first WNV outbreaks in Italy were associated with a wetland area (Romi et al. 2004). However, further spreading during the previous years was not necessarily associated with wetlands, as observed in the United States (Ezenwa et al. 2007). Vectors and host interactions with the virus and environment may determine the disease patterns. Here we found that due to its feeding behavior, *Cx. pipiens* is potentially more important for the enzootic cycle of WNV than *Cx. hortensis*. The abundance of *Cx. pipiens* was positively related to temperature and negatively to rainfall. Although seroconversion was reported in the study area in sentinel chickens and humans, we failed to detect any mosquitoes infected with flavivirus. Therefore, our data suggest a silent cycle at low enzootic transmission levels probably driven by *Cx. pipiens*.

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