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1. BACKGROUND

The genus *Flavivirus* (family Flaviviridae) is comprised of more than 70 viruses that, according to their mechanism of transmission, are included in one of the following three groups: 1) those infecting a range of vertebrate hosts through mosquito (MBV) or tick (TBV) bites, called "arthropod-borne viruses"; 2) those spread without a known vector (UNKV), presumed to be limited to infecting vertebrates only, and 3) those apparently limited to insects alone, called "insect-specific flaviviruses" (ISFs) (Ishikawa & Konishi, 2011; Huhtamo *et al.,* 2012 and references herein).

The *Flavivirus* genome contains gene coding for three structural proteins (capsid, premembrane and envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) (Lindenbach *et al.,* 2006). For phylogenetic analysis of flaviviruses, regions encoding envelope, NS3 and NS5 are the most frequently used. When considering their observed pathogenicity, those with the highest impact on human and animal health in Europe and abroad belong to the first group, and include West Nile Virus (WNV), Usutu Virus (USUV), Tick-borne Encephalitis Virus, Dengue virus, Japanese encephalitis virus and Yellow fever virus (Gubler *et al.,* 2007; Ishikawa & Konishi, 2011; Huhtamo *et al.,* 2012).

WNV is a zoonotic multi-host pathogen belonging to the Japanese encephalitis sero-complex. Reported for the first time in Uganda in 1937 (Smithburn *et al.*, 1940), it then radiated into Europe, India, Asia, Australia and America. During the last decade, new strains with various pathogenic characteristics, grouped into eight distinct lineages, have been discovered (Pesko & Ebel, 2012; Sambri *et al.*, 2013), and it is now considered the most widespread arbovirus in the world (Weaver & Reisen, 2010). WNV is maintained in nature by a cycle involving ornithophilic mosquitoes as the vector, principally *Culex* spp. (Diptera: Culicidae), and birds that are the amplifying hosts. It infects a broad range of avian and mammalian species, but has also been reported to infect reptiles and amphibians. Some vertebrates including humans and horses, act epidemiologically as "dead end" hosts since they are susceptible to infection but do not transmit the virus (Kilpatrick *et al.*, 2006a). Other mechanisms of transmission include mites and ticks, organ transplant, blood transfusion, breastfeeding, intrauterine infection, and the fecal-oral route (Komar *et al.*, 2003; Lawrie *et al.*, 2004; Zeller & Schuffenecker, 2004;

Kilpatrick *et al.*, 2006b; Blázquez & Sáiz, 2010; Monini *et al.*, 2010) and references therein. WNV infection results in flu-like symptoms or neurological disorders with heavy sequelae and eventually death. Many studies, however, have shown that this virus can circulate silently, infecting animals and humans asymptomatically (Komar *et al.*, 2001; Banet-Noach *et al.*, 2003; Rizzoli *et al.*, 2007; Monini *et al.*, 2010; Sambri *et al.*, 2013).

In Italy, WNV is the flavivirus with the greatest impact on human and animal health. WNV lineage 1 has been circulating since 1998 (Autorino *et al.*, 2002). Surveillance activities established in 15 Italian wetlands from 2001 to 2007 detected only sporadic WNV circulations in several areas through seroconversions in domestic chickens (*Gallus gallus*, hereafter chicken) and horses (Filipponi *et al.*, 2005; Rizzoli *et al.*, 2007; Calistri *et al.*, 2010a and references therein). Since 2008, WNV lineage 1 has been detected in animals, mosquitoes, and humans in an increasing number of Italian Regions each year, with clinical symptoms reported in horses and humans (Monaco *et al.*, 2011). In 2011, the first human infection of WNV lineage 2 was discovered in central Italy, and later detected in birds and mosquitoes in north-eastern Italy and Sardinia (Bagnarelli *et al.*, 2011). Until now, the virus has caused more than 100 cases of human neuroinvasive disease and more than 50 cases of WNV fever (Rizzo *et al.,* 2012; Barzon *et al.,* 2013a; Ciccozzi *et al.*, 2013; IZSAM G.Caporale - Teramo, 2014).

USUV is another emerging pathogenic flavivirus, isolated for the first time from *Culex* (*Cx.*) *neavei* mosquitoes in South Africa in 1959 and since then reported in several African countries (Williams *et al.,* 1964). It is maintained in nature by a mosquito-bird transmission cycle, with the genus *Culex* as the main vector, and for several years it has been considered a virus with very low pathogenicity for humans and animals (Nikolay *et al.*, 2011). It was historically only detected in tropical and subtropical Africa. However, the first European cases were confirmed in Italy in 1996 (Weissenböck *et al.,* 2013) and then in Austria in 2001 (Weissenböck *et al.*, 2002), resulting in the deaths of several species of resident birds, including common blackbirds (*Turdus merula*, hereafter, blackbird), Great gray owls (*Strix nebulosa*) and barn swallows (*Hirundo rustica*) (Weissenböck *et al.*, 2002, 2013). In the following years, more cases were registered in Italy (Rizzoli *et al.*, 2007; Gaibani *et al.*, 2012) and the virus was detected in birds and/or mosquitoes of other several countries, including Austria, Hungary, Switzerland (Steinmetz *et al.*, 2011), Czech Republic, England (Buckley *et al.*, 2006), Spain and

Germany (Becker *et al.*, 2012) in both animals and mosquitoes, with an increasing trend of animal infections (Weissenböck *et al.*, 2002; Lelli *et al.*, 2008; Manarolla *et al.*, 2010; Tamba *et al.,* 2011; Vázquez *et al.,* 2011; Cerutti *et al.*, 2012; Ravagnini *et al.*, 2012; Buchebner *et al.,* 2013; Calzolari *et al.,* 2013a; Höfle *et al.,* 2013). Moreover, the virus appears to have increased in pathogenicity, with fatalities in European wild birds (Steinmetz *et al.*, 2011; Weissenböck *et al.,* 2013). In Europe, USUV has been recognised as candidate human pathogen in Austria (Weissenböck *et al.*, 2007), Italy (Cavrini *et al.*, 2009; Pecorari *et al.,* 2009; Savini *et al.*, 2011; Gaibani *et al.*, 2012, 2013; Pierro *et al.*, 2013) and Croatia (Vilibic-Cavlek *et al.*, 2014), but the pathogenicity for humans still requires further assessment.

The first ISF discovered was the cell-fusing agent virus (CFAV), isolated in 1975 from the culture fluid of a cell line established from the mosquito *Aedes* (*Ae.*) *aegypti* (Stollar & Thomas 1975; Cammisa-Parks *et al.,* 1992). Since then, it has been detected in wild-caught mosquitoes in Puerto Rico (Cook *et al.,* 2006), Thailand (Kihara *et al.,* 2007) and Mexico (Espinoza-Gómez *et al.,* 2011). The inclusion of ISFs in the *Flavivirus* genus is supported by similarities with other flaviviruses in terms of genomic organization, polyprotein hydropathy profiles and cleavage sites, but they are not able to replicate in mammal cells and they have been isolated only in mosquito-derived cells (Kuno, 2007; Hoshino *et al.*, 2009; Bolling *et al.*, 2011; Cook *et al.,* 2012; Haddow *et al.*, 2013). Over the past 40 years, many other ISFs have been isolated and identified from field-collected mosquitoes, belonging to different species, in several locations (Ferreira *et al.*, 2013; Haddow *et al.,* 2013; Papa *et al.*, 2014). These includes Kamiti River virus (KRV), Culex flavivirus (CxFV), Spanish Culex flavivirus (SCxFV), *Culex theileri* flavivirus, Aedes flavivirus (AeFV), *Aedes vexans* flavivirus (AeveFV), Czech *Aedes vexans* flavivirus, *Aedes galloisi* flavivirus, *Aedes cinereus* flavivirus, Ochlerotatus flavivirus (OcFV), Ochlerotatus flavivirus from Portugal ($OcFV_{PT}$), Spanish Ochlerotatus flavivirus (SOcFV), Quang Binh virus, Nakiwogo virus, Calbertado virus, Chaoyang virus and Hanko virus. Integrated sequences or DNA forms related to ISFs have been described in *Ae. aegypti*, *Aedes vexans*, *Aedes albopictus, Ochlerotatus* (*Oc.*) *caspius* and *Ochlerotatus* spp. mosquitoes (Crochu *et al.,* 2004; Cook *et al.,* 2009; Roiz *et al.,* 2009; Calzolari *et al.,* 2010a; Sánchez-Seco *et al.,* 2010; Vázquez *et al.,* 2012; Ferreira *et al.*, 2013; Haddow *et al.,* 2013; Papa *et al.*, 2014). ISFs have also been detected in other insects, such as sandflies in Algeria and Spain

(Moureau *et al.,* 2010; Sánchez-Seco *et al.,* 2010), and as flavivirus-related sequences in adult chironomids in France (Cook *et al.,* 2013). In Italy, several ISFs have been reported in regions in the northern part of the country (Trentino, Veneto, Emilia-Romagna, Lombardia and Piemonte), namely AeFV in *Ae. albopictus* mosquitoes, OcFV in *Oc. caspius* and *Cx. pipiens*, AeveFV in *Ae. vexans* and *Aedes cinereus/geminus* flavivirus in *Ae. cinereus/geminus* (Roiz *et al.,* 2009, 2012a; Calzolari *et al.,* 2010a, 2010b, 2012a, 2013a; Cerutti *et al.,* 2012; Ravagnini *et al.*, 2012; Pautasso *et al.,* 2013). Moreover, DNA sequences related to AeFV were identified in wild *Ae. albopictus* mosquito collected in 2007 (Roiz *et al.,* 2009).

The ecoepidemiology of arboviruses, and flaviviruses in particular, is influenced by several factors that have been under study during the last decades because of their possible implications on determining human and animal risk of infection.

One of these factors are wild birds since they are believed to have the potential to maintain, transport, and disperse several flaviviruses, as reviewed by some authors (e.g.: Pfeffer & Dobler, 2010). Wild birds living in Africa, Europe and Asia can be divided into migratory and non-migratory (or "resident"). The latter permanently live in the territory where they are born and travel only short distances to search for food and new ecosystems. Migratory birds annually undertake journeys, principally in spring and autumn, from their reproductive territory to where they will spend the winter (overwintering grounds) and viceversa. The former include intrapaleartic (or short-distance) migrants moving between Europe, Asia and North Africa; whilst others are long-distance or transaharian migrants, flying between Europe and southern Africa. Since the first appearance of WNV in North America in 1999 (Lanciotti *et al.*, 1999), much research has been carried out to understand the epidemiological role of bird species, demonstrating that migratory birds are implicated in the spread of diseases over long distances, such as from Africa into Europe, while the successive spread at a local level is mainly induced by resident and short-distance migrants, both for WNV and USUV (e.g.: Pfeffer & Dobler, 2010; García-Bocanegra *et al.*, 2011; Steinmetz *et al.*, 2011; Weissenböck *et al.*, 2013 and references therein). At the stopover sites along their migratory route and once they reach their destination grounds, migratory birds share common habitats with resident species from which they are otherwise separated during the rest of the year, and this exposes them to a great range of vectors and pathogens.

The physiological stress of migration can increase their susceptibility to WNV, and/or lead to the reactivation of latent and chronic infections (Hedentröm, 2008; Newton, 2008; Pfeffer & Dobler, 2010 and references therein). Among the non-vectorial transmission routes of WNV between birds, oral and fecal viral shedding plays a central epidemiological role for many reasons. The fecal-oral secretions and excretions can contaminate the environment, leading to a high number of individuals coming into contact with the virus. In addition, this transmission route can take place in several ways, such as direct and indirect contact (e.g.: inhalation of aerosols, ingestion of contaminated food, preening soiled feathers), intra- and inter-species socialization, feeding of the nestlings, cannibalism and scavenging of infected carcasses. In fact, the viremia in orally-infected animals is similar to the one reached after mosquito bites (Komar *et al.*, 2003; Zeller & Schuffenecker, 2004; Rizzoli *et al.*, 2007; Blázquez & Sáiz, 2010; Monini *et al.*, 2010; Reiter, 2010 and references therein). Furthermore, oral and fecal shedding may last longer than the viraemic phase (usually less than 7 days (Komar *et al.*, 2003)), can occur without apparent clinical signs, and may play an important role in determining whether WNV can become established in areas or during seasons when the mosquito densities are too low to provide significant vector-borne transmission (Rizzoli et *al.*, 2007). Oral and fecal shedding and/or oral infection have also been reported in some species of mammals and reptiles (Blázquez & Sáiz, 2010) and references therein.

Very little is currently known about USUV, mainly because it was historically confined to Africa, and because its pathogenicity to humans and animals has only recently been recognised. Moreover, these studies have focused in detecting the virus in dead birds (e.g.: Becker *et al.*, 2012; Weissenböck *et al.*, 2013), through serological tests (e.g.: Buckley *et al.*, 2006; García-Bocanegra *et al.*, 2011) or through virological or biomolecular testing of blood samples (Savini *et al.*, 2011). Fewer studies using oral and cloacal swabs have been carried out to detect USUV (Chvala *et al.*, 2005, 2006), although in another study it was detected in gastrointestinal tract and kidneys of birds using a biomolecular test (Weissenböck *et al.*, 2003).

Another factor potentially influencing the circulation of flaviviruses is the presence of two or more viruses in the same mosquito vector, the so-called “co-infection”. This aspect concerns in particular the co-infection of more viruses belonging to *Flavivirus*

genus. In fact, despite their non-pathogenicity for humans and animals, and their apparent inability to replicate in vertebrate cells, ISFs have recently gained attention with respect to their ecological and evolutionary relationships with other important disease-causing flaviviruses. An important field of research is the analysis of viral co-infections which can lead to different outcomes, such as "super-infection exclusion" or enhanced transmission or replication (Farfan-Ale *et al.,* 2010; Newman *et al.,* 2011; Bolling *et al.,* 2012; Vázquez *et al.,* 2012; Hobson-Peters *et al.,* 2013). Super-infection exclusion has been postulated as a mode of competition for host between related viruses: a mosquito infected with ISFs may be resistant or less competent to arbour and transmit another related virus (Sánchez-Vargas *et al.,* 2004; Lee *et al.,* 2005). In this case, ISFs could provide indirect protection against the transmission of related pathogenic flaviviruses (Bolling *et al.,* 2012; Kenney *et al.*, 2014). However, evidence of super-infection exclusion has not been always reported in case of viral co-infections. For example, Kent *et al.* (2010) found that CxFV Izabal strain did not affect the vector competence of *Cx. quinquefasciatus* for transmitting WNV, when mosquitoes were infected sequentially in the laboratory, and Crockett *et al.* (2012) found no evidence supporting an association between WNV and CxFV infection prevalence in wild mosquitoes.

Arboviruses, as flaviviruses, circulate in enzootic cycles among arthropod vectors and a number of animal species, which act as reservoirs. Each virus requires the contemporary occurrence of competent vertebrate reservoir hosts and mosquito species acting as vectors (Kuno & Chang, 2005). Variables such as climate, habitat structure, and the relative abundance and behaviour of vectors and hosts all contribute to the complexity that characterises the dynamics of transmission of vector-borne pathogens (Kilpatrick, 2011; Reisen, 2013; Rosà *et al.*, 2014; Marcantonio *et al.*, 2015). Spill-over events are the result of complex ecological interactions affecting pathogens, vectors, and their hosts (Weaver & Reisen, 2010). Species-specific variation in both contact rates and infectiousness drives considerable heterogeneity in pathogen transmission (Kilpatrick *et al.*, 2006b). Contact rates depend on two factors, the local composition of species or biodiversity, and the host feeding preference of mosquitoes.

The role played by biodiversity in the epidemiology of viral diseases is still debated because it can vary in any single ecoepidemiologic scenario. The relationships among high host diversity and low virus spillover have been observed in several disease models,

including WNV (Kilpatrick *et al.*, 2006b; Swaddle & Calos, 2008; Ostfeld, 2009; Keesing *et al.*, 2010), indicating that high species diversity may reduce human exposure to vector-borne diseases (Ostfeld & Keesing 2000a, 2000b). A primary mechanism by which biodiversity may moderate disease risk is called the "dilution effect" and may operate for a wide range of vector-borne diseases (Ostfeld & Keesing, 2000a, 2000b; Schmidt & Ostfeld, 2001; Holt *et al.*, 2003; LoGiudice *et al.,* 2003; Telfer *et al.,* 2005). It predicts that infection rates among vectors will be lower in highly diverse host communities where incompetent reservoir hosts diluite rates of disease transmission between vectors and highly competent hosts. Conversely, if species tend to be highly competent reservoirs, high species diversity may actually increase disease prevalence. This opposing effect, called a "rescue effect" by Ostfeld & Keesing (2000b), may describe the relationship between passerine diversity and WNV prevalence if multiple passerine species serve as competent virus hosts (Ezenwa *et al.*, 2006).

The two main factors taken into account when measuring biodiversity are richness and evenness. Richness is a measure of the number of different kinds of organisms present in a particular area. The more species present in a sample, the richer the sample. Evenness compares the similarity of the population size of each of the species present. It's a measure of the relative abundance of the different species living in a certain area (Colwell, 2009).

Biodiversity can be studied using diversity indexes, which are quantitative measures that reflect the number of different kinds of organisms present in a particular area and/or compares the similarity of the population size of each of the species present (Magurran, 2004).

Regarding the host feeding preference, some mosquito species are generalist and express opportunistic feeding behaviour, while others are specialists and feed preferentially on selected hosts (Burkett-Cadena *et al.*, 2008; Farajollahi *et al.*, 2011). Studies of mosquito feeding preference are essential to understand the ecology of arbovirus transmission. In fact, at a population level, such feeding preferences may enhance or reduce transmission if vectors feed on competent or incompetent hosts, respectively (Carver *et al.*, 2009). If vector blood meals occur more commonly on non-competent or relatively less-competent host species, viral replication and transmission will consequently be limited and therefore the circulating viral load within a given

population will decrease over time, undergoing the so-called “dilution effect”. Conversely, if vectors come in contact with a population composed of more-competent hosts, viral replication will be enhanced and the consequent high circulating viral load may lead to spillover events and to the geographical spread of the infection (“amplification effect”) (Ezenwa *et al.*, 2006; Hamer *et al.*, 2011 end references therein). Host feeding preferences vary among mosquito species and populations, and are affected by factors including season, mosquito nutritional status, host behaviour or mosquito learning over time (Kilpatrick *et al.*, 2006a; Hamer *et al.*, 2011; Burkett-Cadena *et al.*, 2012; Thiemann *et al.*, 2012; Takken & Verhulst, 2013; Janousek *et al.*, 2014). Studying the local biodiversity in terms of type and number of species and number of individuals can help understanding the eco-epidemiology of viral diseases.

In Europe, *Culex pipiens s.l.* is the principal vector of WNV and USUV (Vázquez *et al.*, 2011; Di Sabatino *et al.*, 2014 and references therein). This species occurs in two biological forms (biotype), *Cx. pipiens f. pipiens* and *Cx. pipiens f. molestus*, which can hybridize. *Cx. pipiens* biotype is subjected to diapause, is anautogeneous, eurygamous, has a greater ecological plasticity and bites mainly birds, while the *f. molestus* biotype doesn’t diapause, is autogeneous, stenogamous, more restricted to habitats with human influence and bites mammals (Harbach *et al.*, 1984,1985; Vinogradova 2000). In Europe, sympatric occurrence and hybridization of the two biotypes have been observed in aboveground and underground habitats (Reusken *et al.*, 2010; Gomes *et al.*, 2013 and references herein). Since hybrid forms exhibit an opportunistic behaviour and can readily feed on mammals and birds, they are supposed to act as major bridge vector for some flaviviral infections, as WNV, between infected birds, which are the natural reservoir, and other hosts such as humans and domestic mammals (Fonseca *et al.*, 2004; Osório *et al.*, 2013 and references therein). Nevertheless, a study carried out in Portugal (Gomes *et al.*, 2013) found that also the *f. molestus* biotype shows a high ornithophilic tendency, potentially increasing the odds for alternate feeding on birds and mammals and, consequently, the risk of WNV transmission from birds to accidental hosts. Therefore, the relative proportion of the various biotypes may change locally the epidemiology of mosquito borne viruses.

Analyses of the vertebrate origin of the blood meals of wild-caught mosquitoes indicated that *Cx. pipiens s.l.* prefers to feed on certain vertebrate hosts, independently

from their local relative abundance, thus potentially influencing the transmission of avian and mammalian pathogens (Muñoz *et al.*, 2012 and references therein). In the case of WNV, there is evidence that a reduced number of bird species, called "super-spreaders", are responsible for most of the viral replication and transmission, mainly because they are preferred hosts by the mosquito vector and competent hosts for the virus (Kilpatrick *et al.*, 2006b; Paull *et al.*, 2012, Rizzoli *et al.*, 2015). In fact, the importance of each vertebrate host in pathogen transmission depends on the host reservoir competence, defined as the relative ability of a reservoir host species to maintain and transmit the pathogen to a competent vector, but also on contact rates between the host and competent mosquito vectors (the so called "feeding preference"), which is a function of two factors: host relative abundance and mosquito feeding habits (Kilpatrick *et al.*, 2007; Simpson *et al.*, 2012).

Female mosquitoes locate and choose vertebrate hosts at a distance using olfactory, visual, and other cues like temperature (e.g.: the heat of the body host) (Clements, 1999). Vertebrates release volatile compounds produced through their epidermal cells, glands and the metabolic activity of the bacteria colonizing the body surface. These compounds are detected by the insect chemosensory systems and may differ according to species, sex, age and seasonality (Campagna *et al.*, 2012). Differences in odour composition have been shown to be significant in determining host preference in mosquitoes (Lefèvre *et al.,* 2009), and odour extracts have been used to test mosquito host preference (Syed & Leal 2009; Campagna *et al.*, 2012).

Mosquitoes feeding preferences have been studied using different tools such as traps baited with hosts, wind tunnels, choice chambers and dual-choice olfactometers in which mosquitoes can express their preferences by flying toward and landing on a particular host (e.g.: Balenghien *et al.*, 2006; Takken & Verhulst, 2013) or measuring the response of olfactory receptor neurons to constituents of bird and human headspace extracts using electroantennography (Syed & Leal 2009). In order to distinguish between opportunistic and specialized feeding behaviours, blood meal analysis alone is insufficient, as it fails to take into account differences in host availability and behaviour (Thiemann *et al.*, 2012). Recognising this, Hassan *et al.* (2003) proposed a "feeding preference index", which examines the number of blood meals from a given host species as a fraction of blood meals from all identified hosts, and compares them with the

proportional abundance of that species in the host community. By combining this information with choice experiments in the laboratory, it is possible to test preferences in the absence of confounding factors (Lefèvre *et al.*, 2009).

Despite a much longer history of virus circulation in the Old World (Zeller & Schuffenecker, 2004), a detailed understanding of virus ecology and vector-host interactions is still lacking in Europe. Currently, a number of field studies have identified mosquito hosts using blood-meal analysis (e.g.: in Czech Republic (Radrova *et al.*, 2013), Spain (Muñoz *et al.*, 2012), Italy (Roiz *et al.*, 2012b), Portugal (Gomez *et al.*, 2013) and Israel (Valinsly *et al.*, 2014)). However, to my knowledge, there has been no assessment of host preference in Europe either by integrating blood meal analyses with host availability in the field, or by choice experiments in the laboratory. Gomes *et al.* (2013) found that both *Cx. pipiens* biotypes take blood meal from house sparrow (*Passer domesticus*) and blackbird. These bird species frequent human settlements and buildings, and are quite tolerant towards human presence. Given the catholic feeding habits of hybrids and *f. molestus* biotype highlighted in previous studies, the proximity of these two bird species to anthropic environment may increase the spillover transmission of WNV to humans.

Trentino province, is a mountainous region in northern-eastern Italy with approximately 70% of the territory more than 1000 m above sea level, and about 55% covered by coniferous and deciduous forests, with principally a temperate-oceanic climate. The southern part of this territory is located around Lake Garda and it is the only part of Trentino where a sub-Mediterranean climate can be found (Roiz *et al.*, 2011). Trentino is located on many of the short- and long-distance routes of migratory birds that, from northern Europe, cross the Alps on their way to western Asia or Africa and viceversa (Spina & Volponi, 2008a, 2008b). Trentino is bordered to the north by the Alto Adige province which is mountainous and broadly covered with forest as well. Veneto neighbours Trentino at east and south. Apart from its northern portion which is quite similar to Trentino, more than half of the region surface is hilly and flat, with a large plain (Pianura Padana), characterized by mild climate, irrigated areas, wetlands, marshes, medium-small urban settlements, intensive agriculture and animal husbandry with abundant mosquito and bird populations. In Trentino, evidence of WNV and USUV circulation has been detected sporadically through seroconversion of sentinel chickens in

2005, but no evidence of current active virus shedding from birds or occurrence in mosquitoes have been recorded so far (Rizzoli *et al.,* 2007; Grisenti *et al.,* 2013). A completely different eco-epidemiological situation characterises the Veneto region where WNV (Filipponi *et al.,* 2005, 2007; Calistri *et al.,* 2010a; Barzon *et al.,* 2013a, 2013b) and USUV (Busani *et al.,* 2011; Savini *et al.,* 2011; Regione Veneto, 2010, 2013; Engler *et al.,* 2013; Gobbi *et al.,* 2014) have been detected on several occasions during the last 10 years, and now circulate endemically with periodical outbreaks. Similarly, the eco-epidemiology of ISFs seems to differ among these two Italian regions. Specifically, in Trentino, ISFs have been detected in *Ae. albopictus* since 2007 when AeFV DNA sequences were identified from wild-caught *Ae. albopictus* mosquitoes (Roiz *et al.,* 2009). In 2008, the same AeFV was identified and isolated in C6/36 cell cultures, and sequences supposed to be *Aedes cinereus/geminus* flavivirus were detected in one pool of *Ae. cinereus/geminus* mosquitoes (Roiz *et al.,* 2012a). In Veneto the only reported detection of ISFs was DNA sequences from AeFV integrated in the genome of *Ae. albopictus* collected in 2007 (Roiz *et al.,* 2009). The reasons for these disparities in the pattern of flavivirus infection in mosquitoes in north-eastern Italy have not yet been clarified.

2. AIMS AND SCOPE

The aims of this study are:

1) to investigate the possible presence and estimate the prevalence of flaviviruses in two regions of Northeastern Italy, Trentino and Veneto, through the screening of mosquitoes using biomolecular analyses.

2) to evaluate the role of the following factors in the eco-epidemiology of flaviviruses:

- Migratory birds:

In Trentino, only a silent circulation of WNV and USUV has been detected so far, but the animal species involved in this cycle have not yet been determined. Despite WNV and USUV share some ecological characteristics, knowledge of the natural transmission cycle and of the importance of non-vectorial transmission of USUV are still lacking. Due to the strategic position of Trentino in relation to short- and long-distance routes of migratory flyways, and the possible role played by migratory birds in the introduction and dispersion of these two flaviviruses, I carried out a biomolecular survey to detect if active virus shedding occurs in migratory birds captured during their seasonal migrations, and to evaluate the role of different species in spreading these viruses.

- Flavivirus infection pattern in mosquitoes:

Due to increasing reports of ISFs being detected globally, it is important to improve the knowledge on their geographic distribution and ecology, especially to get insight into the potential consequences of ISFs' infection on the vectorial capacity of mosquitoes. The aim of the current study was, therefore, to analyse the pattern of viral infection in mosquitoes collected in Veneto and Trentino, which are characterized by significant differences in ecological conditions and epidemiological patterns of WNV and USUV infections.

- Mosquito host feeding preferences:

In this part of the study, with field data collected in Veneto region, I aimed to quantify feeding preferences of *Cx. pipiens*, considered the principal vector of WNV in Europe. Using two complementary approaches I first identified the feeding preference of *Cx. pipiens* in nature by combining analysis of blood meal origin with assessment of host availability, and I also analysed seasonal and spatial variation in host preference. Then, I analysed the feeding preference in the absence of confounding variables (environmental

variations, host abundance and behaviour) by testing the relative attractiveness of odour extracts from wild birds for a laboratory colony of *Cx. pipiens*.

Moreover, since there is still significant lack of knowledge on the effect of the genetic background of *Cx. pipiens s.l.* mosquitoes and the hybridization between *f. molestus* and *f. pipiens* biotypes on the host selection, I conducted studies on feeding preferences in two genetically different *Cx. pipiens* populations using two complementary approaches. The first one was to study a wild population collected in Trentino characterized by a large prevalence of the *f. pipiens* biotype, by combining analysis of blood meal origin with assessment of host species abundance. In the second one, I tested a lab-colony derived from a population characterized by a large prevalence of the *f. molestus* biotype, by using headspace extract solutions collected from avian host species.

- Biodiversity of species:

The biodiversity index is a non-parametric tool used to describe the relationship between species number and abundance. Assigning biodiversity values at specific sites has been used to describe community composition and structure (Colwell, 2009). Moreover, this parameter can help understanding the ecoepidemiology of diseases with particular regard to infective diseases transmitted by vectors (Schmidt & Ostfeld, 2001; Holt *et al.*, 2003; LoGiudice*et al.,* 2003; Telfer*et al.,* 2005). There are several different indexes with specific merits and properties. As concluded by many researchers (Magurran, 2004), the most satisfactory results can be gained using a combination of different indexes which measure aspects such as species richness, evenness and abundance. For these reasons I tried to investigate the influence of avian and mosquito community in Trentino and Veneto on the flavivirus ecoepidemiology by calculating three biodiversity indexes namely, Simpson's Index, Shannon's Index and Pielou's Index, for data collected in 2011 and 2012.

3. MATERIALS AND METHODS

3.1 STUDY N.1: THE ROLE OF MIGRATORY BIRDS

Bird netting

Sample collection was carried out in Trentino-Alto Adige region during ringing campaigns in autumn 2011 and 2012 (September and October) and spring 2012 (March to May). Intrapaleartic and transaharian migratory birds were captured by ornithologists using net labyrinths authorized by ISPRA (Istituto Superiore per la Protezione e la Ricerca Ambientale, Ozzano dell'Emilia, Bologna, Italy) within the European Union for Bird Ringing (EURING) which includes ethical approval. The research protocol was also approved by the Wildlife Management Committee of the Autonomous Province of Trento (Italy). These activities are carried out to provide data on migration patterns, demography and ecological processes. The sampling sites included: Faedo (Trento) and Tiarno di Sopra (Trento) during the 2011 autumnal ringing campaign; Cloz (Trento), Campi al lago (Caldaro, Bolzano), Campodenno (Trento), Calavino (Trento) and Sarche (Trento) during the 2012 spring ringing campaign; Faedo (Trento), Tiarno di Sopra (Trento) and San Michele all'Adige (Trento) during the 2012 autumnal ringing campaign (Figure 1).

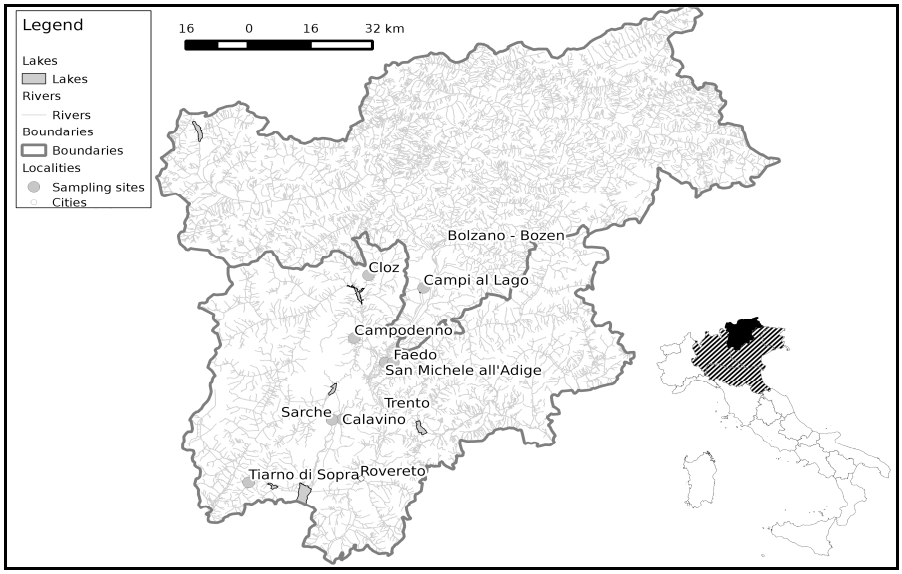

Figure 1: Bird sampling sites. Italian map insert: black area, sampling region of Trentino-Alto Adige; hatched area, neighbouring regions (Veneto, Lombardia, Emilia Romagna, Friuli-Venezia Giulia) with active WNV and USUV circulation.

Sampling

Oral and cloacal samples were taken from each captured bird using sterile swabs with transport medium AMIES without charcoal, in polypropylene tubes Ø 12 × 150 mm (Nuova Aptaca S.r.l., Canelli - Asti, Italy). Samples were kept refrigerated during transport to the laboratory, where they were stored at -80°C until analysis. Each bird was manipulated only for few minutes and prior to its release, each one was marked by standard procedures using metal leg rings, according to EURING procedures. Date of capture, species, ring number, age, weight and other morphobiometric parameters were recorded for each individual.

RNA extraction and Polymerase Chain Reactions (PCRs)

Molecular analyses were performed in the laboratory of the Department of Veterinary Sciences at the University of Torino (Italy). For RNA extraction, each swab was dissolved in 200 μl of phosphate-buffered saline (PBS) buffer (Sigma-Aldrich, Milano, Italy) and the suspension obtained was centrifuged for 5 minute at 8000 rpm. 140 μl of the supernatant was added to 560 μl of Buffer AVL and carrier RNA, prepared according to QIAamp® Viral RNA Mini Handbook (Qiagen, Hilden, Germany). The samples were then processed following this protocol. In the final step, RNA was eluted in 60 μl of Buffer AVE. After quantification with Thermo Scientific Nanodrop 2000 (Thermo-Scientific, Euroclone, Milano, Italy), up to 1 μg of RNA was reverse-transcribed according to QiagenQuantiTect® Reverse Transcription Kit Handbook (Qiagen, Hilden, Germany). For the screening of flaviviruses, a generic nested RT-PCR was used, that amplifies a region of the NS5 gene well-conserved within this genus, according to Sánchez-Seco *et al.* (2005), with modifications (using a volume of 5 μl of the cDNA of the first PCR, 5 U of HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany), 40 pmol of each generic Flavivirus primer (Flavi1+, Flavi1-), and 10 nmol of each dNTP). In the nested PCR mix, 1 μl of PCR product from the first reaction was added to 49 μl of reaction mix composed by 1.25 U of HotStarTaq DNA Polymerase, 40 pmol of each primer (Flavi2+, Flavi2-), and 10 nmol for each dNTP. Finally, the products of the nested PCR were analysed by electrophoresis with a 1.5% (w/v%) agarose gel (Sigma-Aldrich, Milano, Italy) and visualized by staining with 0.1% (w/v%) of ethidium bromide. Positive and negative controls were included in the analyses.

3.2 STUDY N.2: PATTERN OF FLAVIVIRUS INFECTION IN MOSQUITOES

Mosquito collection

Mosquitoes were captured between May and October 2011 and 2012, from Veneto and Trentino (Figure 2). In each region, BG-Sentinel™ traps (BioQuip Products Inc., Rancho Dominguez - CA, USA) were placed in a rural and an peridomestic environment. Once a week, the traps were set up with BG-Lure® attractant (BioQuip Products Inc., Rancho Dominguez - CA, USA) and every two weeks the traps were additionally baited with dry ice as a source of carbon dioxide. Each trap was set up at the morning and checked after 24 hours. In Veneto, the traps (n = 20) were placed in Pianura Padana. Data on WNV occurrence in mosquitoes at each locality was obtained from a regional surveillance program (Gobbi *et al.*, 2014) with three of the sampling localities (6 traps) recorded as WNV positive and the other seven (14 traps) as WNV negative (Figure 3).

In Trentino, the traps (n = 10) were located around Lake Garda (Figure 4) and, in order to collect a high number of blood-engorged mosquitoes, a backpack aspirator was also used to collect blood-fed *Cx. pipiens* females sweeping outdoor vegetation in the areas surrounding mosquito traps, as done in previous studies with similar purpose (Hamer *et al.*, 2009; Simpson *et al.*, 2012).

Captured mosquitoes were killed by placing them at -80°C for 10 minutes and were identified to species level on a chill table using morphological characteristics according to classification keys (Becker *et al.*, 2010; Schaffner *et al.*, 2011). Host-seeking mosquitoes captured were then pooled in eppendorfs according to date, trap, species and gender with a maximum number of 50 individuals per pool. To preserve the samples, a solution composed of EMEM (Minimum Essential Medium Eagle Modified, Safc Biosciences, Milano, Italy) supplemented with FBS (Thermo Scientific Hyclone Inc., Logan- UT, USA) 10% (v/v%) and a mixture of antibiotics (Penicilin 0.5 mg ml-1 and Streptomicine 0.5 mg ml-1, Euroclone) 0.5% (w/v%) was added. If more than 30 mosquitoes were present in these pools, 700 μl of this solution was added; if there were less than 30 mosquitoes, 500 μl was added. The pools were stored at -80°C until analyses. Blood-fed mosquitoes were stored individually at -80°C, in centrifuge tubes with 1 ml of ethyl alcohol 70% until analysis. I carried out flavivirus screening only on mosquitoes collected in 2012 since this sample, in both regions, was bigger and more homogeneous than that collected in 2011.

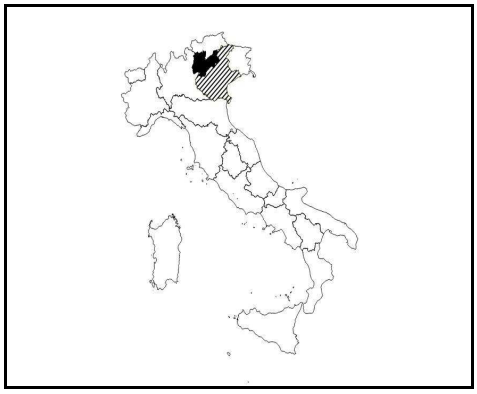

Figure 2: Italian map with mosquito sampling regions highlighted: Trentino (black area); Veneto (hatched area).

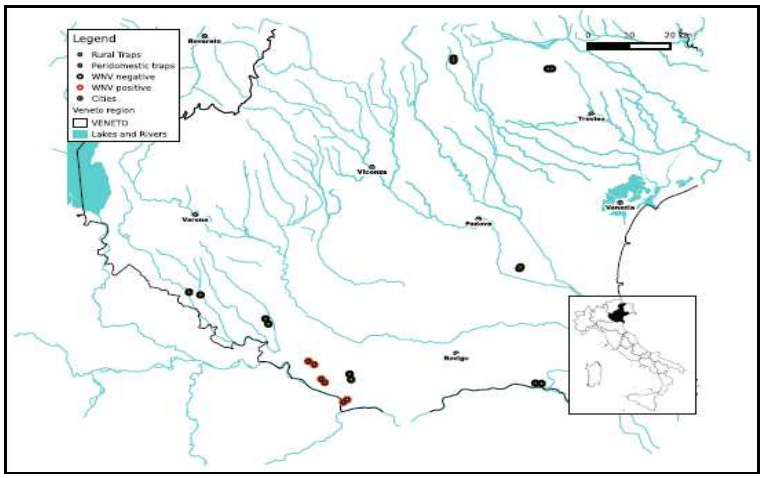

Figure 3: Map of mosquito trapping sites in Veneto region in the period 2011-2012. The lower boundary corresponds to the Po Valley (Pianura Padana). Inset indicates the location of the Veneto region. Positivity for WNV in mosquitoes was recorded in the period 2010-2012.

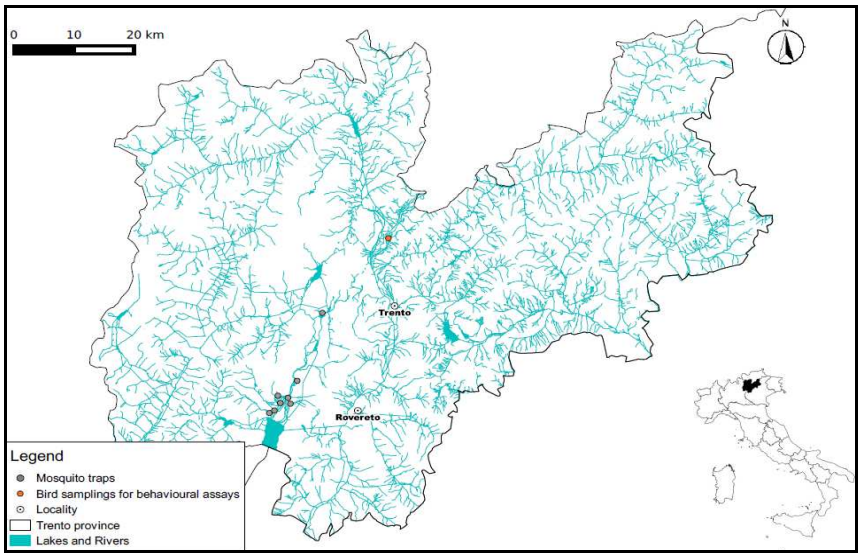

Figure 4: Map of mosquito trapping sites in Trentino region in the period 2011-2012 (grey dots) and sampling site (red dot) for birds used in behavioural assays during the summer 2013 (see later). Inset indicates the location of the Veneto region.

Flavivirus screening of mosquitoes collected in 2012

Viral RNA was extracted from 140 µl of mosquito pools or cell culture supernatants using a QIAamp® Viral RNA Kit (Qiagen, Hilden, Germany). Positive and negative controls were included in the analyses. Flavivirus detection was performed using a generic RT-nested-PCR designed in the NS5 gene, described by Sánchez-Seco *et al.* (2005). RT-PCR was conducted using a One-Step® RT-PCR kit (Qiagen, Hilden, Germany). The samples positive for *Flavivirus* were selected for further analysis by using the RT-nested PCR method described by Vázquez *et al.* (2012). The amplicons of 1019 nt in the NS5 gene obtained with this method contain enough phylogenetic information for taxonomic studies. The final amplified products for both reactions were analysed by electrophoresis on a 1.5% (w/v%) agarose gel (Sigma-Aldrich, Milano, Italy) and visualized by GelRed™ staining (Biotium, Segrate - Milano, Italy). The amplicons were purified using the QIAquickW® PCR Purification Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions and sequenced in both directions. The obtained sequences were compared with those available in public databases.

Cell culture, virus isolation, and electron microscopy studies

For some positive samples virus isolation was attempted in C6/36 cell lines (from *Ae. albopictus* mosquito) and incubated at 33°C. A total volume of 100 µl of the macerated mosquito supernatants were inoculated into 25 cm^2 flasks with C6/36 cells. After absorption for 2 h at 33°C, 5 ml of maintenance medium was added. Cells were observed daily for CPE. The culture supernatants were collected after a minimum of three blind passages and stored at -80°C until tested by RT-PCR. Fresh supernatants and cells from CPE positive cultures were used for electron microscopy studies. The supernatants were fixed at a final concentration of 2% (w/v%) glutaraldehyde, clarified by low-speed centrifugation, ultracentrifugated at 35000 rpm for 60 min in a Ty 50 Ti Beckman rotor at 4°C, and negative stained with 2% (w/v%) neutralized sodium phosphotungstate. The cells' monolayers were fixed with 2% (w/v%) glutaraldehyde, and were put together with the cell pellets from the supernatant clarifications, dehydrated in serial ethanols, and embedded in epoxydic resin for ultrathin sectioning in a Ultracut UC6 ultramicrotome. Viral particles were identified according to their ultrastructural characteristics in a Tecnai 12 or a Philips CM12 electron microscope.

Phylogenetic analysis

The sequences obtained in this study from different species of mosquito were compared with sequences obtained from other members of the genus *Flavivirus* through the NCBI web server using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were assembled and hand-edited using the program SeqMan (DNASTAR software). The multiple sequence alignment Clustal W algorithm within the MEGA version 5 software package (Tamura *et al.,* 2011) was used to obtain an optimal sequence alignment file, with manual adjustment to maintain a correct reading frame. Phylogenetic analyses were conducted on 1055 nt of the NS5 gene with the sequences obtained in this study and other representative flaviviruses, using distance-based neighbour-joining method and distance-p model. The reliability of the inferred neighbour-joining trees was evaluated by bootstrap analysis of 1000 data replicates. The sequences obtained from the large fragment of the NS5 gene, were submitted to public database (GenBank), and the accession numbers are indicated in the phylogenetic tree. USUV strain used in this work for molecular and phylogenetic studies was obtained during a viral mosquito surveillance carried out during the 2011 in Veneto region.

Statistical analysis

Generalized linear models with binomial error distributions were used to assess how AeFV infection was affected by the following explanatory variables: region (Veneto vs. Trentino) and environment (peridomestic vs. rural) of sampling sites, month of sampling (from May to October), mosquito genus and the size of the pool used to test the presence of the virus in mosquitoes. In addition all two-way interaction terms among explanatory variables were included into the full model. Multi-model inference (Burnham & Anderson, 2002) was used to compare all possible models using the R package “MuMIn” (Barton, 2013). Models were ranked using Akaike information criterion (AIC), and differences in AIC (ΔAIC) between consecutively ranked models were used to calculate weights and relative evidence ratios for each variable. The best models were selected using a threshold of $\Delta AIC \leq 2$ (Burnham & Anderson, 2002). All variables included in the best models were ranked according to their importance (weight), and the average coefficient for each variable was calculated.

3.3 STUDY N.3: ROLE OF THE FEEDING PREFERENCE OF MOSQUITOES

Mosquitoes collection

Sample collection was carried out using the protocol already described in chapter 3.2. The large part of blood-fed mosquitoes were collected during the sampling season 2012. For this reason, this study was conducted taking into account field data (mosquito blood meal and bird census) registered in 2012. For mosquitoes collected in Trentino, the digestion status of mosquito blood meals was visually scored by using the Sella score ordinal rating system *S* (Martinez-de la Puente *et al.*, 2013). Generalized linear models (GLMs) with binomial error distribution and logit link function were implemented to test for the effect of the blood meal digestion status on the success of host identification in blood-fed females and on the frequency of specific host species identification.

Molecular identification of *Culex* mosquito biotypes

Mosquitoes collected in 2012 in Trentino and those belonging to the lab colony used to compare the feeding preference founded in laboratory with that founded in field condition in Trentino, were molecularly identified as belonging to the *Cx. pipiens* complex by ACE-assay to distinguish *Cx. pipiens s.l.* and *Cx. torrentium*, following the protocol published by Smith & Fonseca (2004). The same DNAs of *Cx. pipiens s.l.* were further amplified by using CQ11-assay that identifies the *f. pipiens* and *f. molestus* biotypes, and hybrids. The CQ11-assay was chosen as a promising and useful marker to discriminate the biotypes at the population level (Bahnck & Fonseca, 2006).

DNA extraction and identification of blood meal origin

The protocol of Alcaide *et al.* (2009) was followed to identify the vertebrate host species of blood fed female mosquitoes. The abdomen of each mosquito was separated from the head-thorax in Petri dishes using sterile tips and the DNA contained in each abdomen was isolated using the DNeasy Blood and Tissue® kit (Qiagen, Hilden, Germany) following company specifications (see Martìnez-De La Puente *et al.*, 2013). This method improves the identification success of mosquito hosts with respect to other DNA extraction procedures. A nested-PCR protocol that selectively amplifies 758 bp of the vertebrate mitochondrial Cytochrome c Oxidase Subunit I (COI) gene was used (Alcaide *et al.*, 2009). Negative DNA extraction controls were included in PCR reactions. After sequencing of

the amplified COI fragment, the identification engine implemented in the Barcode of Life Data (BOLD) Systems database (http://www.barcodinglife.org/views/idrequest.php) was used to assign COI sequences to particular species.

Census of wild birds around the sites of mosquito traps

Wild bird availability was estimated in 2011 and 2012 from surveys carried out once a month within 5 days of each mosquito-trapping period. Bird counts were made using both sightings and calls. Counts were started at sunrise and conducted for 4 h, and were carried out for 6 min at each of the five locations, these being at the trap site and at points 150-200 m from the mosquito trap site in each cardinal direction. For each observation (visual and auditory), species and number of individuals were recorded. Where additional species were observed outside count periods, records were added to species lists. Since in Trentino engorged mosquitoes were collected principally during August and September 2012, the feeding preference was calculated taking into consideration only census data registered during those months.

Calculation of mosquito feeding preferences on avian hosts

Data on avian host abundance and mosquito feeding habits were used to compute feeding preference indexes (P_i) of mosquitoes, defined as:

Feeding preference index $$\boxed{P_i = \frac{f_i}{a_i}} \quad (1)$$

where f_i represents the fraction of total blood meals taken by *Cx. pipiens s.l.* from host *i* (feeding habits) and a_i represents the density of species *i* over the total density of the avian community (Hassan *et al.*, 2003). If mosquitoes feed opportunistically on host species in proportion to their abundance, the fraction of blood meals from each species, f_i, will be the same as the fraction of the community made up by that species, a_i, and P_i will therefore be 1. $P_i < 1$ and $P_i > 1$ represent avoidance or preference for that species, respectively. Several species present in the avian host community were not detected in blood meal samples. For those species it was necessary to determine whether this absence was due to avoidance, or to insufficient sample size. For those species, a value $f_0 = (1\text{-}0.5^{1/b})$ was assigned, which represents half the probability of not observing any

blood meals from this species given the total blood meal sample size, *b*. Then, for species that were not detected in mosquito blood meals, I assumed a conservative estimate $P_i = f_0/a_i$ if the species was significantly avoided or $P_i = 1$ if not. Following the approach of Kilpatrick *et al.* (2006), by means of multinomial simulations, was tested whether P_i for each species was significantly different than 1. Specifically, the distribution of blood meals between species obtained with 10.000 simulations was compared with those expected under the null hypothesis of opportunistic feeding habits, i.e. that *Cx. pipiens s.l.* fed on hosts proportionally to their abundance.

Sample size constraints of data available for Trentino region, allowed to carry out further statistical analyses only on data collected in Veneto. In particular, in order to compare the pattern of mosquitoes feeding habits in peridomestic and rural sites, two different feeding preference indices as in (1) were computed using blood meals and avian census data obtained with traps in peridomestic, $P_{i.peridomestic}$, and rural, $P_{i.rural}$, localities, respectively. To test the significance of these differences, multinomial simulations were used where samples of blood meals and host species abundances in each simulation were extractions from multinomial distributions with probabilities $f_{i.peridomestic}$ and $a_{i.peridomestic}$ in peridomestic sites and $f_{i.rural}$, and $a_{i.rural}$, in rural sites, respectively (where $f_{i.peridomestic}$ [$f_{i.rural}$,] represents the fraction of total blood meals taken by *Cx. pipiens* from host *i* in peridomestic [rural] sites and $a_{i.rural}$, [$a_{i.rural}$,] represents the density of species *i* over the total density of the avian community estimated in peridomestic [rural] sites). For each host species the probability of observing a larger [or smaller] feeding preference index in peridomestic than in rural sites was estimated by computing the fraction of the 10,000 simulations where the difference in feeding preferences indices, $P_{i.peridomestic} - P_{i.rural}$,, was positive [or negative]. Similarly, in order to investigate the seasonal patterns of mosquito feeding habits, two different feeding preference indexes as in (1) were computed by using blood meals and avian census data obtained in the early (May-June, $P_{i.early}$) and the late (July-September, $P_{i.late}$) mosquito activity season. These two periods were selected in order to test whether mosquito feeding habits are affected by the seasonal changes in the behaviour of some avian species. For instance, frugivorous birds, such as blackbird, at the end of its breeding season (in July), moves from nesting areas to sites rich in fruit bearing plants (Snow & Snow, 1988; Berthold, 2001). Other species, such as barn swallow (*Hirundo rustica*), after the breeding season

move for gregarious foraging or start migrating (Snow & Perrins, 1998). These behavioural changes modify the composition of the avian host community and are therefore likely to affect the feeding patterns of *Cx. pipiens*. Finally, the same method was used to compare feeding preference indices between sites where WNV occurrence in mosquitoes has, or has not, been observed ($P_{i.WNV+}$, or $P_{i.WNV-}$, respectively) during 2010-2012 (Gobbi *et al.*, 2014). Simulations were performed using MATLAB 7.10.0 (The Mathworks, Inc.).

Rearing of *Cx. pipiens s.l.* mosquitoes

Culex pipiens s.l. mosquitoes used in the behavioural assays to study the feeding preference for both Trentino and Veneto regions were derived from a population collected as larvae in an above-ground puddle placed in Cafferella Park, a wide urban park in Rome (Central Italy). The mosquitoes were reared for several generations in the Insectarium of Infectious, Parasitic and Immune-Mediated Diseases Department of Istituto Superiore di Sanità (Rome, Italy). Mosquitoes were maintained under specific environmental parameters (26 ± 1 °C, 70% R.H., 14 h:10 h (L:D) photoperiod). Eggs, larvae and pupae were reared in a 3‰ (w/v%) solution of sodium chloride in distilled water and fed with fish flakes until adulthood. Male and female mosquitoes were bred in the same cage in order to allow mating and maintained with sugar solution (10% w/v%). To obtain egg-rafts and in order to exclude any possible bias that could affect the results of the behavioural experiments, the mosquito-colony was reared under laboratory conditions for several generations, feeding on both mammals and birds. In summer 2013, the colony was transferred and reared in a climatic chamber (Proclimatic, Imola - Bologna, Italy) of the laboratory of the Department of Biodiversity and Molecular Ecology of the Fondazione Edmund Mach (San Michele all'Adige - Trento, Italy), using the same laboratory environmental parameters. Only female mosquitoes of F9 generation were used in the behavioural assays.

For the part of the study concerning the comparison between the feeding preference detected in the field with that resulting from lab testing, a representative sample of mosquitoes employed (n = 40 specimens) were molecularly analysed using CQ11-assay (already described above).

Odour collection from wild birds

In Trentino, the blood meal analysis carried out on wild mosquitoes in this study (see Results section, chapter 4.3), as well as previous field studies carried out in this (Roiz *et al.*, 2012) and other European areas (Gomes *et al.*, 2013; Rizzoli *et al.*, 2015), showed that most of the wild-caught *Cx. pipiens s.l.* mosquitoes had fed on blackbirds and house sparrows. For these reasons, I tested the feeding preference of the *Cx. pipiens* lab-colony for the dominant species in term of blood meals, above mentioned, using the headspace extract solutions (odour) collected from their bodies. Moreover, I also tested the attractiveness of some common but not highly abundant species, spotted flycatcher (*Muscicapa striata*), European robin (*Erithacus rubecula*), song thrush (*Turdus philomenos*), and humans.

However, I could not test Eurasian tree sparrows (*Passer montanus*) since they are very difficult to capture as their population density is very low, living in more localized and rural areas than house sparrows.

With respect to Veneto region, based on the outcome of blood meal analysis and field census of this study (see Results section, chapter 4.3), four wild bird species were selected: blackbird and the Eurasian magpie (*Pica pica*, hereafter magpie), both abundant and preferred in the field; house sparrow, abundant and fed on opportunistically, and Eurasian blackcap (*Sylvia atricapilla*, hereafter blackcap), neither abundant nor preferred but displaying feeding and breeding habits similar to blackbird.

Differences in odour composition were previously shown to be significant in determining host preference in mosquitoes (Lèfevre *et al.*, 2009), and odour extracts was used to test mosquito host preference (Syed & Leal, 2009; Campagna *et al.*, 2012; Whittaker *et al.*, 2013). Following these studies, in the current research odour extracts were used rather than live birds.

Bird captures were carried out during spring and summer of 2013 using bird-nets in agricultural lands located in the municipality of San Michele all'Adige (Trento, Italy - Figure 4), near the laboratories where the survey was carried out. Captures were carried out by an ornithologist authorized by ISPRA and the research protocol was approved by Local Wildlife Management and Veterinary Welfare Committees. Since sex and age may influence the composition of odour bouquet emitted by birds and, consequently, the mosquito feeding preference (Campagna *et al.*, 2012; Takken & Verhulst, 2013), I

collected the odour from four adult men and captured four individuals of each of the following host categories: house sparrow adult male, blackbird adult male, blackbird adult female, blackbird young male, blackbird young female, blackcap adult male, magpie adult male, spotted flycatcher adult male, European robin adult male and song thrush adult male.

Each captured bird was placed into a paper box and immediately transported to the laboratory, preserving its welfare conditions. It was collocated in an airtight polypropylene dessicator (Carlo Erba Reagents, Milano, Italy) of diameter 140 mm (for house sparrows, European robins and blackcaps) or 240 mm (for blackbirds, spotted flycatchers, song trushes and magpies) according to the size of the bird. Charcoal-filtered air was pumped through the system at 150 mL min^{-1} and over a Porapak Q cartridge that contained 50 mg of adsorbent (Sigma-Aldrich, Milano, Italy) for 1 h for each animal (Anfora *et al.*, 2009). Afterwards the birds were immediately released at the site of capture. The headspace extracts were desorbed by eluting the cartridge with 600 μl of redistilled hexane (Carlo Erba Reagents, Milano, Italy) and stored at -20 °C until used. To avoid cross contaminations among birds, the dessicator was cleaned with denatured alcohol between each use. Odour collection from men was realised considering only one hand and forearm, in order to have a surface comparable with bird's ones. To avoid odours contamination, the volunteers were asked to take away watch, rings and bracelets and to clean the hand and the forearm with some denatured alcohol before collocating them in a 25 × 38 cm polyacetate bag (Toppits, Melitta, Sweden). Charcoal-filtered air was pumped through the same system used for birds, for 1 h for each volunteer. Volatiles were desorbed and stored following the protocol above described.

Behavioural assays

The behavioural assays were conducted in August and September 2013 during the peak of host-seeking activity of *Cx. pipiens s.l.* (about 2 h after sunset) (Montarsi *et al.*, 2015) in a room with infrared light to mimic the environmental conditions of the crepuscular-nocturnal activity of this species (Balenghien *et al.*, 2006; Reddy *et al.*, 2007). The assays were carried out in a plastic Petri dish (diameter 25 cm, height 4 cm), that was used as the test arena, placed in a white and uniformly illuminated box (50 cm x 30 cm, 100 lux) at equal distance from the center, in order to prevent mosquitoes from being distracted

by the surrounding objects (Figure 5). The bottom of the dish was covered with a filter paper disk. Two small Petri dishes (diameter 2 cm), with at the bottom a piece of filter paper (1 cm^2) each, were placed inside the bigger Petri dish on two opposite sides: one was soaked with 40 μl of a headspace extract solution of the different categories of *Cx. pipens s.l.* hosts, whereas the other one was soaked with 40 μl of redistilled hexane, thus acting as control. Before experiments, the small Petri dishes were kept for 10 min in a climatic chamber (25 ± 2 °C and 60 ± 5% R.H.) in order to allow solvent evaporation. The test arena was split into three equal areas: one lateral sector including the odour extract, a 5 cm wide central strip, and one lateral sector including the control. For each host category, the test was repeated using headspace extract solutions obtained from four different individuals. Since statistical analysis (see Results section, chapter 4.3) showed that, among the birds species tested, house sparrows and blackbirds were the least and the most attractive to the mosquitoes respectively, in a series of test the attractiveness of these two extracts were compared. In addition, preliminary analyses were carried out to test for positional bias, by conducting trials first with both filter papers soaked with hexane, and then both soaked with odour extract (in this case, from blackbirds), in each case with 30 mosquitoes. Female mosquitoes, individually collected from the cage using a mouth aspirator, were inserted through a little hole of the lid of the Petri dish and observed for 7 min. For each test 100 mosquitoes were observed. The time spent in each of the sectors was recorded. Mosquitoes that remained in one of the lateral sectors for at least 70% of the test duration were scored as having a preference. Mosquitoes that spent less than 70% of the time in either lateral sector or remained in the central sector demonstrated no preference.

For each host category, the number of mosquito specimens that made a choice on the odour source was compared with that choosing the control sector. For each test, the positions of the disks of filter paper were randomly assigned to avoid any position effect. For all the tests, individual mosquitoes were used only once to avoid bias from previous exposure.

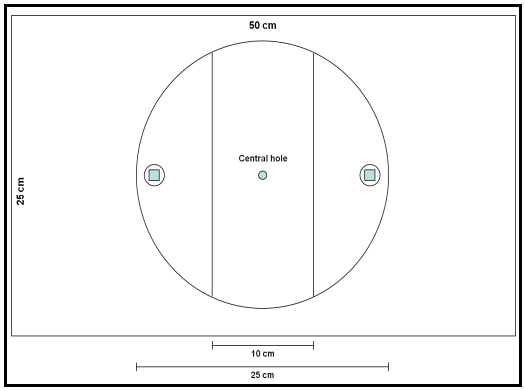

Figure 5: Graphic representation of the arena used for the behavioural assays.

Calculation of odour preference

For each host species, a Chi-square (χ^2) test was used to compare the number of mosquito specimens that chose the odour source versus those that chose the control sector within each bioassay. Individuals exhibiting no preference were excluded. Differences among host categories were evaluated by contingency table analysis based on χ^2 followed by a Ryan's multiple comparison test on proportions ($p < 0.05$) (Ryan, 1960). Both χ^2 tests were Yates corrected.

3.4 STUDY N.4: STUDY OF BIODIVERSITY WITH DIVERSITY INDEXES

Using the data on mosquito sampling and bird censuses collected during 2011 and 2012, and reported in chapter 4.3 and Tables 2, 6, 7, 11 and 12. The following biodiversity indexes were calculated:

Shannon's Index (H') $$H_{SH} = -\sum_{i=1}^{S} p_1 log_2 p_1 \quad (2)$$

Simpson's Index (S') $$H_{SI} = 1 - \sum_{i=1}^{S} (p_1)^2 \quad (3)$$

Pielou's Index (J') $$J' = \frac{H'}{\ln S} \quad (4)$$

Shannon's Index (2) takes into account the number of taxa, that in the specific case of this study are mosquito and bird species, and the relative abundance of each taxa in the community under study, which in this case are Trentino and Veneto regions. The higher the number of species and individuals living in a community, the higher the value of this index. Simpson's Index (3) is also called Index of Dominance since it indicates if in a certain community one or few taxa outnumber (or dominate) the other taxa. The higher its value, the higher the number of individuals belonging to one or few species compared to the other species. Pielou's Index (4) accounts only for the relative abundance of taxa so its value increases if the number of individuals of a certain species is equivalent to the number of individuals belonging to the other species.

4. RESULTS

4.1 STUDY N.1: THE ROLE OF MIGRATORY BIRDS

A total of 43 birds were captured during the autumn of 2011, 176 during spring 2012, and 103 during autumn 2012 (Table 1).

Table 1: Number of individuals of the bird species tested in Trentino-Alto Adige in 2011 and 2012. [a]Each species was classified as intrapaleartic (S) and transaharian (L).

Bird species		Family	Order	Migratory pattern[a]	2011 Autumn	2012 Spring	2012 Autumn	Total
Scientific name	**Common name**							
Otus scops	European Scops Owl	*Strigidae*	*Strigiformes*	L	-	-	1	1
Cuculus canorus	Common Cuckoo	*Cucilidae*	*Cuculiformes*	L	-	1	-	1
Jynx torquilla	Eurasian Wryneck	*Picidae*	*Piciformes*	L	-	1	-	1
Aegithalos caudatus	Long tailed Tit	*Egitalidae*	*Passeriformes*	S	-	2	2	4
Lanius collurio	Red backed Shrike	*Lanidae*	*Passeriformes*	L	-	5	1	6
Delichon urbicum	Common House Martin	*Irundinidae*	*Passeriformes*	L	-	-	5	5
Emberiza schoeniclus	Reed Bunting	*Emberizidae*	*Passeriformes*	S	-	3	-	3
Prunella modularis	Dunnoch	*Prunellidae*	*Passeriformes*	S	2	2	-	4
Anthus trivialis	Tree Pipit	*Moracillidae*	*Passeriformes*	L	-	-	1	1
Ficedula hypoleuca	Pied Flycatcher	*Muscicapidae*	*Passeriformes*	S	-	4	6	10
Muscicapa striata	Spotted Flycatcher	*Muscicapidae*	*Passeriformes*	L	-	12	-	12
Sylvia borin	Garden Warbler	*Silvidae*	*Passeriformes*	L	-	9	1	10
Sylvia curruca	Lesser Whitethroat	*Silvidae*	*Passeriformes*	S	-	-	3	3
Hippolais polyglotta	Melodius Warbler	*Silvidae*	*Passeriformes*	L	-	2	-	2
Hippolais icterina	Icterin Warbler	*Silvidae*	*Passeriformes*	L	-	2	-	2
Acrocephalus scirpaceus	Reed Warbler	*Silvidae*	*Passeriformes*	L	-	15	-	15
Acrocephalus palustris	Marsh Warbler	*Silvidae*	*Passeriformes*	L	-	5	-	5
Acrocephalus arundinaceus	Great reed Warbler	*Silvidae*	*Passeriformes*	L	-	6	-	6
Sylvia atricapilla	Eurasian Blackcap	*Silvidae*	*Passeriformes*	S	1	23	1	25
Locustella naevia	Grashopper Warbler	*Silvidae*	*Passeriformes*	L	-	1	-	1
Sylvia melanocephala	Sardinian Warbler	*Silvidae*	*Passeriformes*	S	-	1	-	1
Phylloscopus trochilus	Willow Warbler	*Silvidae*	*Passeriformes*	L	-	10	5	15
Phylloscopus collybita	Common Chiffchaff	*Silvidae*	*Passeriformes*	S	-	9	-	9
Phylloscopus sibilatrix	Wood Warbler	*Silvidae*	*Passeriformes*	L	-	2	-	2
Periparus ater	Coal Tit	*Paridae*	*Passeriformes*	S	-	-	7	7
Parus major	Graet Tit	*Paridae*	*Passeriformes*	S	-	1	-	1
Phoenicurus phoenicurus	Common Redstart	*Turdidae*	*Passeriformes*	L	-	2	2	4
Phoenicurus ochruros	Black Redstart	*Turdidae*	*Passeriformes*	S	-	-	3	3
Oenanthe oenanthe	Nothern Wheatear	*Turdidae*	*Passeriformes*	S	-	-	1	1
Turdus merula	Common Blackbird	*Turdidae*	*Passeriformes*	S	6	6	15	27
Erithacus rubecula	European Robin	*Turdidae*	*Passeriformes*	S	9	33	24	66
Turdus viscivorus	Mistle Thrush	*Turdidae*	*Passeriformes*	S	1	-	-	1
Turdus philomenos	Song Thrush	*Turdidae*	*Passeriformes*	S	10	9	18	37
Turdus iliacus	Redwing	*Turdidae*	*Passeriformes*	S	-	-	1	1
Luscinia megarhynchos	Rufus Nightingale	*Turdidae*	*Passeriformes*	L	-	6	-	6
Fringilla coelebs	Chaffinch	*Fringillidae*	*Passeriformes*	S	7	2	3	12
C. coccothraustes	Hawfinch	*Fringillidae*	*Passeriformes*	S	4	2	-	6
Carduelis spinus	Siskin	*Fringillidae*	*Passeriformes*	S	1	-	3	4
Fringilla montifringilla	Brambling	*Fringillidae*	*Passeriformes*	S	2	-	-	2
Total					**43**	**176**	**103**	**322**

Among the 39 species captured, 18 were long-distance (L) migratory, and 21 short-distance (S) migratory species. Oral and cloacal swabs taken from each individual captured all tested negative for flaviviruses. The positive control tested always positive, and the negative one resulted always negative.

4.2 STUDY N.2: PATTERN OF FLAVIVIRUS INFECTION IN MOSQUITOES

Species and number of mosquitoes collected in Trentino and Veneto in 2011 and 2012 are reported in Table 2. For the 2011 the table comprehends all the mosquitoes collected (engorged, males and host seeking females). For the 2012 the table reports only the males and host seeking females because the engorged mosquitoes collected are described in detail in the next chapter 4.3.

In particular, in 2012 from the Veneto region a total of 53551 mosquitoes were collected of which 52102 host seeking females, 1190 males, and 259 engorged females belonging to *Oc. geniculatus*, *Oc. caspius*, *Cx. pipiens*, *Cx. territans*, *Cx. modestus*, *Culiseta annulata*, *Anopheles* (*An.*) *maculipennis complex*, *An. plumbeus*, *Ae. albopictus*, *Ae.vexans*, *Ae. cinereus/geminus* and *Ae. koreicus* species. Host-seeking female mosquitoes were divided in 1452 pools, and males into 144 pools. I selected 374 of the female pools for *Flavivirus* screening (total number of mosquitoes = 12266) belonging to *Oc. geniculatus*, *Oc. caspius*, *Cx. pipiens*, *Cx. territans*, *Cx. modestus*, *An. maculipennis complex*, *Ae. albopictus*, *Ae. vexans* and *Ae. koreicus* species. In total, 9.8 % (37/374) of these pools tested positive for *Flavivirus* (Table 3). The sequences detected in this work were grouped in three clusters; two belonging to ISFs and one to the mosquito-borne flavivirus group (Figure 6). In *Cx. pipiens* captured in the town of Erbè (Verona province, GPS 45.241530, 10.969546), one sequence related to USUV was detected. The attempts to amplify a longer fragment to carry out the phylogenetic analysis and the isolation in Vero and C6/36 cells were negative. In order to gain better insight into the evolutionary relationship of circulating USUV, molecular characterization and phylogenetic analysis were performed on one strain detected in the same place in 2011. This USUV was isolated on C6/36 cells and 1000 nt were amplified showing a 100% homology with the Italian USUV strain detected in Bologna in 2009 (USUV_Blackbird_JF266698).

Thirty-five sequences related to AeFV were detected in *Cx. pipiens* (n = 1) and *Ae.*

albopictus (n = 34) mosquito pools and they shared a 97% identity in 1048 nt with AeFV strains detected previously in Italy during 2007 and 2008 in *Ae. albopictus*. The viral isolation in C6/36 cells of 17 positive samples was tried but only the AeFV from one pool of *Ae. albopictus* was successfully isolated, which showed a moderate cytopathic effect (CPE) at 5-7 days post-infection (cell aggregation) as previously described for this group of viruses (Parreira *et al.,* 2012; Vázquez *et al.,* 2012). Viral RNA was successfully amplified from the supernatant at 7 days post-infection using the method described by Vázquez *et al.* (2012) and flavivirus-like particles, enveloped virions of approximately 50-60 nm in diameter, were seen by transmission electron microscopy, in both infected cells and the supernatant of the cell culture (Figure 7).

Finally, a new sequence grouped in a new cluster in the ISFs group was found in another pool of *Cx. pipiens*, whose BLAST analysis in 739 nt shared a 60% similarity with the flavivirus-like sequence described recently from adult chironomids captured in France in 2011 (Genbank accession number KF298267). Genetic distance analysis between this sequence and the rest of the ISF sequences showed an identity of 59%, 56%, 55% and 52% comparing with KRV, CFAV, CxFV and AeFV, respectively. These data suggest that this sequence is potentially divergent from the ISFs already known and could be considered as a new strain (Figure 6). The attempt of isolating the virus inoculating the sample into C6/36 cells failed.

To test whether the positive pools were the result of genomic RNA amplification or DNA forms, some nucleic acid extracts from each group were selected and treated with RNase A (Sigma-Aldrich, Milano, Italy) before amplification without the previous retro-transcription step (Sánchez-Seco *et al.*, 2010). RNase treatment failed to amplify a flavivirus product, suggesting that the sequences obtained were most likely derived from RNA, probably of viral origin.

In Trentino, in 2012 I collected 123 engorged females (*Cx. pipiens* =86; *Ae. albopictus* = 29; *Cx. hortensis* = 8) and a total of 2235 mosquitoes of which 1771 host seeking females, divided in 142 pools, and 464 male mosquitoes divided in 66 pools belonging to *Oc. geniculatus*, *Cx. pipiens*, *Cx. hortensis*, *Ae. albopictus*, *An. maculipennis complex* and *An. plumbeus* species (Table 2 and 3). I selected 124 pools of host seeking female mosquitoes (total number of mosquitoes = 1431) and 14 male pools (total number of mosquitoes = 28) for *Flavivirus* screening, belonging to *Oc. geniculatus*, *Cx. pipiens*, *Cx. hortensis*, *Ae.*

albopictus and *An. maculipennis complex* species. In total, two pools of *Cx. pipiens* and 60 pools of *Ae. albopictus* were positive for AeFV (50%, 62/124) (Table 3). The sequences of these AeFV were similar to the sequences of AeFV detected in mosquitoes from Veneto (Figure 6). Viral isolation was attempted from six *Ae. albopictus* positive pools, which were inoculated into C6/36 cell cultures and the virus was isolated from three of them. In two pools, starting from 3-4 days post inoculation, evident CPE (cellular detachment from the monolayer and the characteristic cell aggregation) was obtained, rhabdovirus-like and flavivirus-like particles respectively were observed by transmission electron microscopy in the cell culture and AeFV viral RNA was detected in the supernatant by RT-PCR (Dietzgen & Kuzmin, 2012; Parreira *et al.,* 2012; Vázquez *et al.,* 2012). No flaviviruses were detected in *Oc. geniculatus*, *Cx. hortensis* and *An. maculipennis complex* collected in Trentino, nor in *Oc. caspius*, *Cx. territans*, *Cx. modestus*, *An. maculipennis complex*, *Ae. vexans*, *Oc. geniculatus* and *Ae. koreicus* collected in Veneto. AeFV prevalence in *Cx. pipiens* and *Ae. albopictus* was calculated, taking into account the number of mosquitoes present in each analysed pool (Table 4).

Regarding the factors influencing AeFV prevalence, mosquito genus was found to be statistically significant, and *Culex* spp. individuals were less infected than *Aedes* spp. individuals. Regarding the geographic disparities, mosquitoes from Veneto were less infected than mosquitoes collected in Trentino, despite the mosquito density being higher in Veneto (Table 5). Co-infection with ISFs and other flaviviruses was not detected in any of the pools examined.

In total, 99 sequences (37 from Veneto and 62 from Trentino) were obtained in this study from pools of different species of mosquitoes. The phylogenetic analysis performed on this partial NS5 gene, showed that the sequences were grouped in three different clusters (Figure 6): one of them contained sequences of AeFV detected from *Ae. albopictus* and *Cx.pipiens* mosquitoes collected in Veneto and Trentino; another one was from a new sequence detected in a pool of *Cx. pipiens* from Veneto; and one was a sequence of USUV detected in *Cx. pipiens* from Veneto. Representative nucleotide sequences obtained in this study from the three different groups of sequences reported in this article have been submitted to GenBank data bank (KM871198, KM871199, KM871200, KM871201 and KM871202 numbers).

Table 2: Number of mosquitoes collected in Trentino and Veneto in 2011-2012.

Mosquito species/mosquito sex	Veneto			Trentino			
	2011	2012		2011		2012	
	female	female	male	female	male	female	male
Oc. geniculatus	*na*	4	0	3	0	4	0
Oc. caspius	*na*	16890	21	0	0	0	0
Cx. pipiens	*2366*	30799	108	150	26	335	13
Cx. territans	*na*	1	0	0	0	0	0
Cx. modestus	*15*	36	0	0	0	0	0
Cx. hortensis	*na*	0	0	28	28	26	21
An. maculipennis complex	*na*	1760	15	7	1	15	0
Ae. albopictus	*na*	2506	1045	377	191	1389	430
Ae. cinereus/geminus	na	1	0	0	0	0	0
Ae. vexans	na	83	0	0	0	0	0
Ae. koreicus	na	11	1	0	0	0	0
An. plumbeus	na	3	0	2	0	1	0
Cs. annulata	na	1	0	1	0	0	0
Oc. spp	na	7	0	0	0	1	0
Total	**2381**	**52102**	**1190**	**568**	**246**	**1771**	**464**

Table 3: Number of mosquito pools and specimens analysed and flavivirus positive in Veneto and in Trentino. AeFV: Aedes flavivirus, ISF: Insect-specific flavivirus, USUV: Usutu virus.

Mosquito species	Veneto	Trentino		
	N. of pools analysed (N. and sex of specimens analysed)	N. of positive pools (AeFV/USUV/New ISF; N. of specimens in the pools)	N. of pools analysed (N. and sex of specimens analysed)	N. of positive pools (AeFV; N. of specimens in the pools)
Oc. geniculatus	2 (2 female)	0	1 (3 female)	0
Oc. caspius	43 (1669 female)	0	-	-
Cx. pipiens	237 (8998 female)	3 (1 AeFV - 1998 female), (1 USUV - 50 female), (1 New ISF - 8 female)	45 (13 male, 214 female)	2 (AeFV - 2 female)
Cx. territans	1 (1 female)	0	-	-
Cx. modestus	8 (18 female)	0	-	-
Cx. hortensis	-	-	8 (15 male, 12 female)	0
An. maculipennis complex	6 (206 female)	0	5 (9 female)	0
Ae. albopictus	65 (1296 female)	34 (AeFV - 25 female)	65 (1193 female)	60 (AeFV - 1095 female)
Ae. vexans	9 (65 female)	0	-	-
Ae. koreicus	3 (11 female)	0	-	-
Total	**374 (12266 female)**	**37 (2056 female)**	**124 (1431 female, 28 male)**	**62 (1097 female)**

Table 4: AeFV prevalence in *Ae. albopictus* and *Cx. pipiens* in Veneto and Trentino regions.

Region	Mosquito species	AeVF prevalence (%)	IC % (low level, upper level)
Veneto	*Ae. albopictus*	3.12	2.07, 4.5
	Cx. pipiens	0.01	0, 0.05
Trentino	*Ae. albopictus*	16.84	12.18, 22.74
	Cx. pipiens	0.88	0.15, 2.69
All regions	*Ae. albopictus*	8.07	6.4, 10.04
	Cx. pipiens	0.03	0.01, 0.08

Table 5: Importance, coefficient estimate and significance of explanatory variables remaining in the best selected models for predicting AeFV infection in mosquitoes (reference levels are: Trentino for Region, Aedes for Mosquito Genus and Rural for Environment).

	Importance	Coefficient Estimate	St.Err.	z value	Pr(>|z|)
(Intercept)		2.93	0.76	3.89	<0.001
Region_Veneto	1	-4.03	0.85	4.72	<0.001
Mosquito_Genus Culex	1	-6.48	0.76	8.52	<0.001
Mosquito_Genus Ochlerotatus	1	-20.98	1525.25	0.01	0.98
Mosquito_Genus Anopheles	1	-21.85	3063.25	0.007	0.99
Pool size	1	-0.02	0.03	0.65	0.51
Pool size:Region_Veneto	1	0.08	0.03	2.16	<0.05
Environment_peridomestic	0.32	0.33	0.46	0.71	0.47

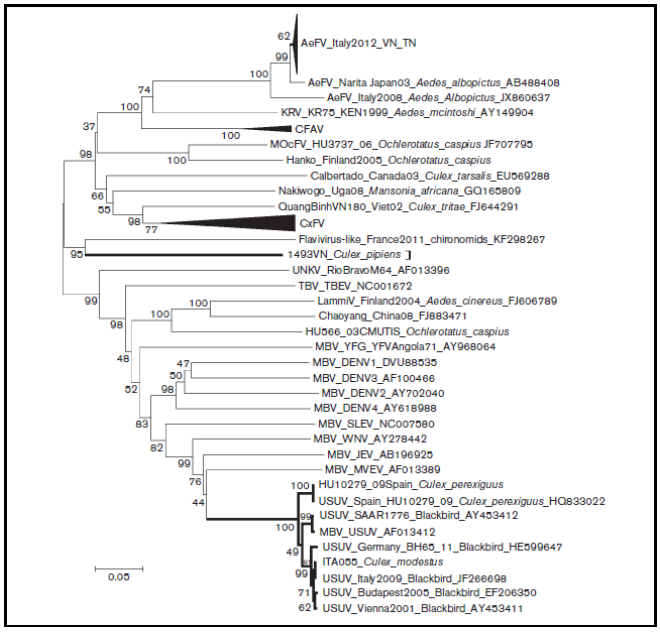

Figure 6: Phylogenetic relationships between positive samples from Veneto and Trentino region and other representative flavivirus sequences, based on 915 nt of the NS5 gene. The tree was constructed using the software package Mega 5.05, neighbor-Joining method and distance-*p* model with 1000 bootstrap replicates. The branches for flavivirus sequences published in the current study (AeFV Italy 2012 VN_TN, 1493TN_*Culex_pipiens* and ITA055 *Culex_modestus*) are in bold. GenBank/EMBL/DDBJ accession numbers for representative sequences obtained in this work are KM871198-KM871202. MBV, Mosquito-borne virus; TBV, tick-borne virus; AEFV, Aedes flavivirus; KRV, Kamiti River virus; CxFV, Culex flavivirus; CFAV, Cell fusing agent virus.

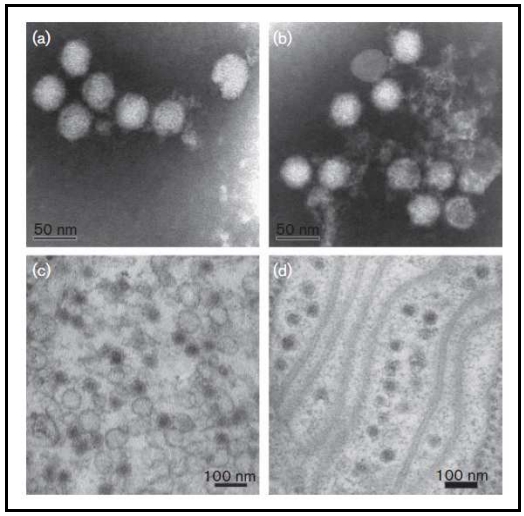

Figure 7: Electron micrographs of C6/36 cells infected with AeFV and USUV. (a/b) Sodio phosphotungstate-negative staining of whole flavivirus-like particles in culture supernatant: USUV (a) and AeFV b). (c/d) Ultramicrotomy. Thin section of infected cells -flavivirus-like particles in cytoplasm: (c) USUV and (d) AeFV.

4.3 STUDY N.3: ROLE OF THE FEEDING PREFERENCE OF MOSQUITOES

- TRENTINO

Mosquito collection and census of wild birds around mosquito trap sites

Host-seeking mosquitoes (species and number) collected during the study period are described in Table 2. During the same period 86 *Cx. pipiens* blood-fed females, 29 *Ae. albopictus* blood-fed females and 8 *Cx. hortensis* were also collected. Results of avian census realised in 2011 are described in Table 6. Avian censuses carried out during spring and summer 2012 showed a total of 75 bird species, including 4507 individuals (Table 7). As stated above, since in Trentino engorged mosquitoes were collected principally during August and September 2012, the feeding preference was calculated taking into consideration only census data registered during those months because they represented the effective availability of birds hosts for the biting mosquitoes. Consequently, only 56 bird species, including 1807 individuals were considered in the statistic analysis. Five of these 56 species dominated the bird community making up about 57% of the total number of individuals: house sparrow, rock dove (*Columba livia*), common starling (*Sturnus vulgaris*), European serin (*Serinus serinus*), and European greenfinch (*Chloris chloris*).

Table 6: Bird species and number of individuals censused in Trentino in 2011.

Family	Common name	Scientific name	N. of individuals
Accipitridae	Short-toed snake Eagle	*Circaetus gallicus*	1
	Common Buzzard	*Buteo buteo*	1
	Eurasian Sparrowhawk	*Accipiter nisus*	1
	Black Kite	*Milvus migrans*	3
Aegithalidae	Long tailed Tit	*Aegithalos caudatus*	10
Alcedinidae	Common Kingfisher	*Alcedo atthis*	3
Anatidae	Wild Mallard	*Anas platyrhynchos*	78
	Domestic Goose	*Anser anser f. domestica*	20
	Common Pochard	*Aythya ferina*	7
	Tufted Duck	*Aythya fuligula*	55
Apodidae	Common Swift	*Apus apus*	87
	Alpine Swift	*Apus melba*	62
Ardeidae	Grey Heron	*Ardea cinerea*	60
Certhiidae	Short–toed Treecreeper	*Certhia brachydactyla*	4
Cettidae	Cetti's Warbler	*Cettia cetti*	10
Cinciclidae	White–throated Dipper	*Cinclus cinclus*	2
Columbidae	Rock dove	*Columba livia*	141
	Eurasian Collared dove	*Streptopelia decaocto*	124
	Turtle dove	*Streptopelia turtur*	2
Corvidae	Eurasian Jay	*Garrulus glandarius*	68
	Common Raven	*Corvus corax*	4
	Hooded Crow	*Corvus cornix*	59
Cuculidae	Common Cuckoo	*Cuculus canorus*	3
Emberizidae	Rock Bunting	*Emberiza cia*	1
	Ortolan Bunting	*Emberiza hortulana*	1
Falconidae	Common Kestrel	*Falco tinnunculus*	3
Fringillidae	Common Chaffinch	*Fringilla coelebs*	208
	European Goldfinch	*Carduelis carduelis*	89
	European Greenfinch	*Cloris chloris*	268
	European Serin	*Serinus serinus*	160
Hirundinidae	Common House Martin	*Delichon urbicum*	214
	Barn Swallow	*Hirundo rustica*	54
Laniidae	Red-backed Shrike	*Lanius collurio*	2
Laridae	Black–headed Gull	*Chroicocephalus ridibundus*	16
Motacillidae	Tree Pipit	*Anthus trivialis*	3
	Western yellow Wagtail	*Motacilla flava*	1
	White Wagtail	*Motacilla alba*	144
	Grey Wagtail	*Motacilla cinerea*	4

Muscicapidae	European Robin	*Erithacus rubecula*	13
	Common Nightingale	*Luscinia megarhynchos*	3
	Spotted Flycatcher	*Muscicapra striata*	24
	Black Redstart	*Phoenicurus ocruros*	1
	Common Redstart	*Phoenicurus phoenicurus*	58
	Winchat	*Saxicola rubetra*	1
	African Stonechat	*Saxicola torquatus*	3
Paridae	Eurasian Blue Tit	*Cyanistes caeruleus*	18
	European Crested Tit	*Lophophanes cristatus*	1
	Great Tit	*Parus major*	79
	Coal Tit	*Periparus ater*	2
	Marsh Tit	*Poecile palustris*	33
Passeridae	Eurasian Tree Sparrow	*Passer montanus*	84
	House Sparrow	*Passer domesticus*	560
Phalacrocoracidae	Great Cormorant	*Phalacrocorax carbo*	11
	Eurasian Wryneck	*Jynx torquilla*	2
Picidae	Great spotted Woodpecker	*Dendrocopos major*	1
	European green Woodpecker	*Picus viridis*	29
Podicipedidae	Great Crested Grebe	*Podiceps cristatus*	1
Rallidae	Eurasian Coot	*Fulica atra*	10
	Common Moorhen	*Gallinula chloropus*	1
Regulidae	Common Firecrest	*Regulus ignicapilla*	3
Scolopacidae	Common Sandpiper	*Actitis hypoleucos*	1
Sturnidae	Common Starling	*Sturnus vulgaris*	33
Sylviidae	Eurasian Reed Warbler	*Acrocephalus scirpaceus*	2
	Melodious Warbler	*Hippolais polyglotta*	1
	Eurasian Blackcap	*Sylvia atricapilla*	218
	Common Chiffchaff	*Phylloscopus collybita*	6
	Willow Warbler	*Phylloscopus trochilus*	1
	Western Bonelli's Warbler	*Phylloscopus bonelli*	2
Turdidae	Common Blackbird	*Turdus merula*	341
	Song Thrush	*Turdus philomelos*	4
Upupidae	Hoopoe	*Upupa epops*	2
Total number of individuals			**3524**
Total number of species			**72**

Table 7: Bird species censused in Trentino in 2012.

Family	Common name	Scientific name	N. of individuals
Aegithalidae	Long tailed Tit	*Aegithalos caudatus*	9
Alcedinidae	Common Kingfisher	*Alcedo atthis*	2
Anatidae	Wild Mallard	*Anas platyrhynchos*	57
	Domestic Goose	*Anser anser f. domestica*	22
	Common Pochard	*Aythya ferina*	9
	Tufted Duck	*Aythya fuligula*	116
	Muscovy Duck	*Cairina moschata*	8
	Mute Swan	*Cygnus olor*	1
Apodidae	Common Swift	*Apus apus*	74
	Alpine Swift	*Apus melba*	5
Ardeidae	Little Egret	*Egretta garzetta*	1
	Grey Heron	*Ardea cinerea*	103
Certhiidae	Short–toed Treecreeper	*Certhia brachydactyla*	1
Cinciclidae	White–throated Dipper	*Cinclus cinclus*	11
Columbidae	Rock dove	*Columba livia*	344
	Common Wood Pigeon	*Columba palumbus*	4
	Eurasian Collared dove	*Streptopelia decaocto*	98
	Turtle dove	*Streptopelia turtur*	1
Corvidae	Eurasian Jay	*Garrulus glandarius*	45
	Common Raven	*Corvus corax*	1
	Hooded Crow	*Corvus cornix*	44
	Carrion Crow	*Corvus corone*	4
Cuculidae	Common Cuckoo	*Cuculus canorus*	2
Emberizidae	Rock Bunting	*Emberiza cia*	3
Fringillidae	Common Chaffinch	*Fringilla coelebs*	189
	European Goldfinch	*Carduelis carduelis*	151
	European Greenfinch	*Cloris chloris*	231
	European Serin	*Serinus serinus*	240
Hirundinidae	Common House Martin	*Delichon urbicum*	254
	Barn Swallow	*Hirundo rustica*	41
	Eurasian Crag Martin	*Ptynoprogne rupestris*	1
Laridae	Black–headed Gull	*Chroicocephalus ridibundus*	2
	Yellow–legged Gull	*Larus michahellis*	1
Motacillidae	Tree Pipit	*Anthus trivialis*	1
	White Wagtail	*Motacilla alba*	99
	Grey Wagtail	*Motacilla cinerea*	10
Muscicapidae	European Robin	*Erithacus rubecula*	3
	European Pied Flycatcher	*Ficedula hypoleuca*	7
	Common Nightingale	*Luscinia megarhynchos*	7
	Spotted Flycatcher	*Muscicapra striata*	41
	Common Redstart	*Phoenicurus phoenicurus*	43
	Winchat	*Saxicola rubetra*	2

	African Stonechat	*Saxicola torquatus*	1
Numididae	Helmeted Guineafowl	*Numida meleagris*	18
Paridae	Eurasian Blue Tit	*Cyanistes caeruleus*	21
	European Crested Tit	*Lophophanes cristatus*	3
	Great Tit	*Parus major*	83
	Coal Tit	*Periparus ater*	8
	Marsh Tit	*Poecile palustris*	8
Passeridae	Eurasian Tree Sparrow	*Passer montanus*	105
	House Sparrow	*Passer domesticus*	852
Phalacrocoracidae	Great Cormorant	*Phalacrocorax carbo*	7
Phasianidae	Domestic Chicken	*Gallus gallus*	110
	Common Pheasant	*Phasianus colchicus*	1
	Common Quail	*Coturnix coturnix*	28
	Turkey	*Meleagris gallopavo*	6
	Black Woodpecker	*Dryocopus martius*	1
	Eurasian Wryneck	*Jynx torquilla*	5
	European Green Woodpecker	*Picus viridis*	23
Podicipedidae	Great Crested Grebe	*Podiceps cristatus*	3
	Little Grebe	*Tachybaptus ruficollis*	8
Rallidae	Eurasian Coot	*Fulica atra*	13
Regulidae	Common Firecrest	*Regulus ignicapilla*	5
Sittidae	Eurasian Nuthatch	*Sitta europaea*	1
Sturnidae	Common Starling	*Sturnus vulgaris*	217
Sylviidae	Cetti's Warbler	*Cettia cetti*	15
	Eurasian Reed Warbler	*Acrocephalus scirpaceus*	3
	Melodious Warbler	*Hippolais polyglotta*	3
	Eurasian Blackcap	*Sylvia atricapilla*	233
	Garden Warbler	*Sylvia borin*	2
	Sardinian Warbler	*Sylvia melanocephala*	5
	Common Chiffchaff	*Phylloscopus collybita*	1
	Western Bonelli's Warbler	*Phylloscopus bonelli*	2
Turdidae	Common Blackbird	*Turdus merula*	417
	Song Thrush	*Turdus philomelos*	11
Total number of individuals			**4507**
Total number of species			**75**

Calculation of mosquito feeding preferences for avian hosts

The 29 *Ae. abopictus* blood-fed females resulted to have fed on *Homo sapiens* (n = 26), blackbird (n = 1), European hedgehog *Erinaceus europaeus* (n = 1) and Eurasian tree sparrow (n = 1). The 8 *Cx. hortensis* blood-fed females fed on Common wall lizard *Podarcis muralis* (n = 7) and *Homo sapiens* (n = 1). Considering the aim of the study, further analyses were carried out and feeding preference indices were computed only for

Cx. pipiens, taking into account the 86 engorged *Cx. pipiens s.l.* mosquito females collected. In particular, 66 of them registered a Sella-score $S = 2$, corresponding to a very fresh blood meal, while the number of fed females registering Sella-scores with $S = 3, 4, 5, 6$ were 1, 1, 14, 4 respectively. The origin of *Cx. pipiens s.l.* blood meals was identified for 66 (76.7%) blood fed females. For the other 20 engorged *Cx. pipiens s.l.*, the blood was too degradated to allow successful DNA amplification and sequencing. Specifically, the success of host identification decreased as the digestion status of the blood meal increased ($\chi^2 = 18.52$, df = 1, $p < 0.0001$). Sixty-four blood meals out of 66 (97%) derived from birds; specifically, 40 from blackbird, 16 from house sparrow, five from Eurasian tree sparrow, one from rock dove, Eurasian collared dove (*Streptopelia decaocto*) and Eurasian wryneck (*Jynx torquilla*). One blood meal was derived from reptiles (common wall lizard) and one from humans. The probabilities to identify blackbird and house sparrow in blood meal samples were not affected by the digestion status of the blood meal (blackbird: $\chi^2 = 0.00024$, df = 1, $p = 0.986$; house sparrow: $\chi^2 = 0.0153$, df = 1, $p = 0.902$). For other species it was not possible to assess the effect of digestion status on the frequency of host species identification due to limited blood meals obtained from these species. Blackbirds contributed to 4.7% of the avian community, but represented 62.5% of the avian blood meals. House sparrows contributed to 18.8% of the avian community, but represented 25% of the avian blood meals. Eurasian tree sparrow contributed to 2.2% of the avian community, but represented 8% of the avian blood meals (Figure 8). The a_i and f_i values were used to calculate the feeding preference index, P_i, for all the avian species detected in *Cx. pipiens s.l.* blood meals (Figure 9). Blackbirds ($P_i = 13.43$, CI = [10.89,15.82], $p < 0.0001$) and Eurasian tree sparrows ($P_i = 3.56$, CI = [1.18,7.78], $p = 0.011$) were significantly preferred by *Cx. pipiens s.l.*. On the other hand, mosquitoes fed on house sparrow ($P_i = 1.33$, CI = [0.84,1.92], $p > 0.05$), Eurasian collared dove ($P_i = 0.94$, CI = [0.024,5.40], $p > 0.05$), and Eurasian wryneck ($P_i = 29.22$, CI = [0.75,167.37], $p > 0.05$) in proportion to their local abundance. Among opportunistic hosts, Eurasian wryneck displayed large uncertainty in feeding preference index estimate because of the limited number of data available in both avian census and blood meals. Rock dove ($P_i = 0.11$, CI = [0.0029,0.65], $p = 0.0012$) was significantly avoided. Moreover, three species were not detected in blood meals despite their high abundance in the avian community, suggesting that these species were significantly avoided (i.e. $P_i < 1$);

they were common starling (p = 0.0012), European serin (p = 0.008), and European greenfinch (p = 0.0134).

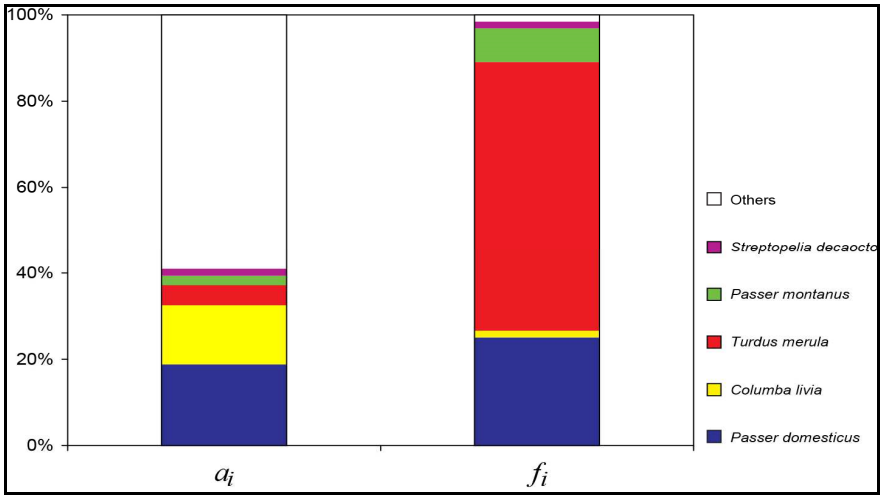

Figure 8: Relative abundance of birds (a_i) and percentage of *Cx. pipiens s.l.* blood meals coming from bird species (f_i) at the study sites in Trentino.

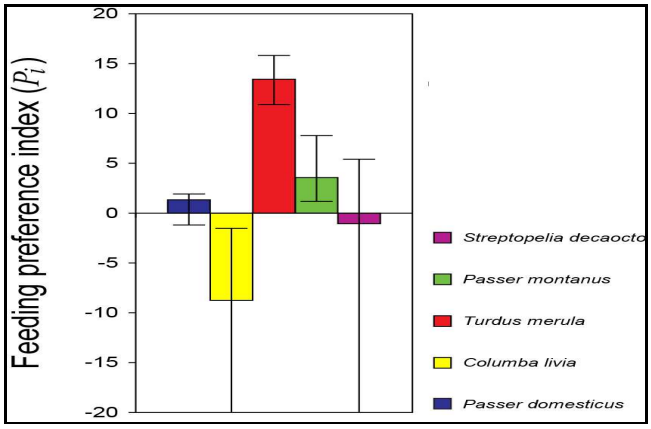

Figure 9: Feeding preference indexes (P_i) of *Cx. pipiens s.l.* mosquitoes and 95% confidence interval of the most notable bird species in Trentino. Positive values indicate preferences; negative values designate avoidance and are calculated as ($-1/P_i$).

Molecular identification of *Culex* mosquito biotypes

In order to genetically characterize the *Cx. pipiens* wild population from the field study area, 68 wild fed-females were analysed using the CQ11-assay. No *Cx. torrentium* was recorded in the sample and overall, the *Cx. pipiens s.l.* genotyping showed the sympatric occurrence of the two biotypes and the hybrids at different frequencies. In particular 85.3% (n = 58) of the specimens were identified as *Cx. pipiens f. pipiens*, 7.3% (n = 5) as *Cx. pipiens f. molestus*, and 7.3% (n = 5) as hybrid. Biotype analyses of the 64 females that fed on birds identified 84.4% (n = 54) as *f. pipiens* biotype, 7.8% (n = 5) as *f. molestus* biotype, and 7.8% (n = 5) as hybrids. Humans and common wall lizards were fed on only

by *Cx. pipiens f. pipiens* biotype. On the other hand, molecular analysis on a representative sample (n = 40) of the lab-colony used in the behavioural assays showed the presence of *Cx. pipiens f. molestus* biotype (67.5%), *Cx. pipiens f. pipiens* biotype (7.5%), and hybrids (25%).

Behavioural assays

A summary of the results of behavioural assays is shown in Tables 8, 9 and 10. The control assay carried out soaking either both filter papers with the solvent (hexane) or both with the headspace extract solutions collected from blackbirds demonstrated the absence of biases (hexane: χ^2 = 0.043, df = 1, p = 0.83; blackbirds: χ^2 = 0.07, df = 1, p = 0.80). Among bird species, the statistical analyses ranked house sparrow and blackbird as the least and the most attractive species, respectively. In Ryan's test house sparrow remained the least preferred species, although not significantly different from magpie, and blackbird remained the most attractive ones although not significantly different from spotted flycatcher, European robin and song thrush, that were all statistically the most attractive (p < 0.001). Humans resulted statistically not attractive (p > 0.05).

All the headspace extract solutions collected from blackbird adult males were attractive to *Cx. pipiens s.l.* compared to the control (χ^2 = 26.9, df = 1, p < 0.001) (Table 9). On the other hand, neither significant differences between adult females nor juveniles versus the control were found (blackbird adult females: χ^2 = 1.3, df = 1, p = 0.22; blackbird young males: χ^2 = 0.3, df = 1, p = 0.53; blackbird young females: χ^2 = 0.1, df = 1, p = 0.83) (Table 1). The comparison in the attraction of the blackbird headspace extract solutions of adult males, young males, adult females and young females to *Cx. pipiens s.l.* showed that adult male blackbirds extract is significantly more attractive than all the other odour sources (young and adult female blackbirds: Ryan's test, p < 0.05: A). Young male blackbirds, young female blackbirds and adult female blackbirds were less attractive, with no significant differences between them (Ryan's test, p < 0.05: B; contingency table χ^2 = 26.5, df = 4, p < 0.001; Ryan's test p < 0.05). Moreover, when tested simultaneously adult male blackbirds extracts were preferred to adult male house sparrows extracts (χ^2 = 6.3, df = 1, p < 0.01) (Table 2). In addition, the *Cx. pipiens* colony used in these experiments showed to be well adapted to laboratory conditions, feeding on mammals and birds and laying both autogenous and anautogenous eggs.

Table 8: Olfactory responses of *Cx. pipiens s.l.* females to headspace extracts of different host species in controlled assays in Petri dish. Columns description. N (%) odour: number and percentage of mosquitoes that exhibited preference to the odour extract solution; N(%) control: number and percentage of mosquitoes that exhibited preference to the solvent; χ^2 statistics: χ^2 test comparing the proportion of mosquitoes choosing odour vs. control for each classes; Ryan's test: comparison of proportions of mosquitoes choosing the odour coming from different hosts (rows with the same letter indicate that proportions are not statistically different at 0.05 level).

Host species	N (%) odour	N (%) control	χ^2	df	p (χ^2)	Ryan's test
House sparrow	35 (64.8)	19 (35.2)	4.16	1	<0.05	A
Spotted flycatcher	35 (79.5)	9 (20.5)	14.21	1	<0.001	C
European robin	39 (75)	13 (25)	12.02	1	<0.001	C
Eurasian blackcap	49 (66.2)	25 (33.8)	7.15	1	<0.01	B
Song thrush	42 (76.4)	13 (23.6)	19.76	1	<0.001	C
Common Blackbird	39 (90.7)	4 (9.3)	26.88	1	<0.001	C
Eurasian Magpie	34 (65.4)	18 (34.6)	4.33	1	<0.05	A
Men	26 (60.5)	17 (39.5)	1.48	1	>0.05	

Table 9: Olfactory responses of *Cx. pipiens s.l.* females to headspace extracts of different age and sex classes of blackbird in controlled assays in Petri dish. Columns description. N (%) odour: number and percentage of mosquitoes that exhibited preference to the odour extract solution; N(%) control: number and percentage of mosquitoes that exhibited preference to the solvent; χ^2 statistics: χ^2 test comparing the proportion of mosquitoes choosing odour vs. control for each classes; Ryan's test: comparison of proportions of mosquitoes choosing the odour coming from different classes (rows with the same letter indicate that proportions are not statistically different at 0.05 level).

Age and gender classes of tested blackbirds	N (%) odour	N (%) control	χ^2	df	*p* (χ^2)	Ryan's test
Adult male	39 (90.7)	4 (9.3)	26.9	1	< 0.001	A
Young male	42 (46.7)	48 (53.3)	0.3	1	0.53	B
Adult female	54 (56.3)	42 (43.7)	1.3	1	0.22	B
Young female	46 (51.1)	44 (48.9)	0.1	1	0.83	B

Table 10: Olfactory responses of *Cx. pipiens s.l.* females to headspace extracts of adult males of blackbird and house sparrow in controlled assays in Petri dish. Columns description. N (%) blackbird: number and percentage of mosquitoes that exhibited preference to the odour extract solution of blackbird; N(%) house sparrow: number and percentage of mosquitoes that exhibited preference to the odour extract solution of house sparrow; χ^2 statistics: χ^2 test comparing the proportion of mosquitoes choosing blackbird vs. house sparrow.

N (%) Blackbird	N (%) House sparrow	χ^2	df	*p* (χ^2)
54 (64.3)	30 (35.7)	6.3	1	< 0.01

- VENETO

Results of avian census realised in 2011 are described in Table 11. Censuses carried out in 2012 showed a total of 31 wild avian species, including over two thousand individuals (Table 12). Eight species dominated the bird community, representing more than 90% of the total number of individuals (Figure 10). They were (from the most to the least abundant) barn swallow, Eurasian collared dove, common starling, house sparrow, rock dove, blackbird, common house martin (*Delichon urbicum*) and magpie.

Table 11: Bird species censused in Veneto in 2011.

Family	Common name	Scientific name	N. of individuals
Accipitridae	Common Buzzard	*Buteo buteo*	1
Ardeidae	Grey Heron	*Ardea cinerea*	5
	Little Egret	*Egretta garzetta*	4
Columbidae	Rock dove	*Columba livia*	224
	Eurasian Collared dove	*Streptopelia decaocto*	231
	Common Wood Pigeon	*Columba palumbus*	2
Corvidae	Eurasian Magpie	*Pica pica*	44
	Hooded Crow	*Corvus cornix*	19
Fasianidae	Common Pheasant	*Phasianus colchicus*	3
Fringillidae	Common Chaffinch	*Fringilla coelebs*	1
	European Goldfinch	*Carduelis carduelis*	15
	European Greenfinch	*Cloris chloris*	2
	European Serin	*Serinus serinus*	2
Hirundinidae	Common House Martin	*Delichon urbicum*	15
	Barn Swallow	*Hirundo rustica*	139
Laridae	Yellow-legged Gull	*Larus michahellis*	6
Meropidae	European Bee-eater	*Merops apiaster*	7
Paridae	Eurasian Blue Tit	*Cyanistes caeruleus*	2
	Great Tit	*Parus major*	14
Passeridae	House Sparrow	*Passer domesticus*	84
Picidae	European Green Woodpecker	*Picus viridis*	6
Psittacidae	Rose-ringed Parakeet	*Psittacula krameri*	1
Rallidae	Common Moorhen	*Gallinula chloropus*	6
Scolopacidae	Common Sandpiper	*Actitis hypoleucos*	2
Sittidae	Eurasian Nuthatch	*Sitta europaea*	1
Sturnidae	Common Starling	*Sturnus vulgaris*	139
Sylviidae	Eurasian Blackcap	*Sylvia atricapilla*	3
Turdidae	Common Blackbird	*Turdus merula*	38
Upupidae	Hoopoe	*Upupa epops*	2
Total number of individuals			**1118**
Total number of species			**29**

Table 12: Bird species censused in Veneto in 2012.

Family	Common name	Scientific name	N. of individuals
Anatidae	Wild Mallard	*Anas platyrhynchos*	9
	Anas spp.	*Anser anser*	3
Apodidae	Common Swift	*Apus apus*	2
Ardeidae	Little Egret	*Egretta garzetta*	7
	Grey Heron	*Ardea cinerea*	2
Charatridae	Northern Lapwing	*Vanellus vanellus*	3
Ciconidae	White Stork	*Ciconia ciconia*	2
Columbidae	Rock dove	*Columba livia*	242
	Common Wood Pigeon	*Columba palumbus*	1
	Eurasian Collared dove	*Streptopelia decaocto*	471
Corvidae	Eurasian Magpie	*Pica pica*	53
	Hooded Crow	*Corvus cornix*	34
Falconidae	Common Kestrel	*Falco tinninculus*	5
Fringillidae	Common Chaffinch	*Fringilla coelebs*	12
	European Goldfinch	*Carduelis carduelis*	10
	European Serin	*Serinus serinus*	9
Hirundinidae	Common House Martin	*Delicum urbicum*	74
	Barn Swallow	*Hirundo rustica*	437
Laridae	Yellow–legged Gull	*Larus michahellis*	11
Muscicapidae	Common Nightingale	*Luscinia megarhynchos*	7
Paridae	Great Tit	*Parus major*	50
Passeridae	House Sparrow	*Passer domesticus*	207
Picidae	European Green Woodpecker	*Picus viridis*	22
Rallidae	Common Moorhen	*Gallinula chloropus*	1
Recurvirostridae	Stilt	*Himantopus himantopus*	5
Sittidae	Eurasian Nuthatch	*Sitta europaea*	2
Sturnidae	Common Starling	*Sturnus vulgaris*	456
Sylviidae	Eurasian Blackcap	*Sylvia atricapilla*	6
	Garden Warbler	*Sylvia borin*	2
Turdidae	Common Blackbird	*Turdus merula*	104
	Common redstart	*Phoenicurus phoenicurus*	3
Total number of individuals			**2252**
Total number of species			**31**

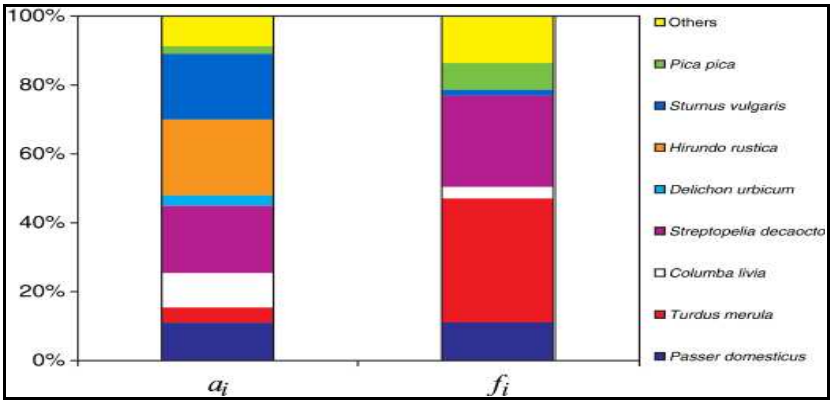

Figure 10: Relative abundance of birds (a_i) and percentage of *Cx. pipiens* blood meals from bird species (f_i) at site traps in Veneto.

In total 259 blood-fed females were collected, identified as *Cx. pipiens* (n = 206), *An. maculipennis comple*x (n = 39), *Ae. albopictu*s (n = 12) and *Oc. caspiu*s (n = 21). The *An. maculipennis comple*x engorged females resulted to have fed on bovine *Bos taurus* (n = 3), dog *Canis lupus familiaris* (n = 6), goat *Capra hircus* (n = 2), rock dove (n = 1), donkey *Equus asinus* (n = 2), horse *Equus caballus* (n = 4), cat *Felis silvestris* (n = 1), chicken (n = 7), *Homo sapiens* (n = 7), European hare *Lepus europaeus* (n = 3), common pheasant *Phasianus colchicus* (n = 1), pig *Sus scrofa* (n = 1), fox *Vulpes vulpes* (n = 1). The *Ae. albopictu*s engorged females resulted to have fed on European hedgehog (n = 1) and *Homo sapiens* (n = 11). The *Oc. caspiu*s engorged females have fed on dog (n = 1), bovine (n = 1), cat (n = 8), donkey (n = 3), horse (n = 3), chicken (n = 2), *Homo sapiens* (n = 3).

Feeding preference indices were computed only for *Cx. pipiens* as sample sizes for other mosquito species were insufficient. A total of 188 hosts of 31 different species were identified from *Cx. pipiens* blood meals. Of these, 144 (77%) were avian of which 117 (62%) were wild birds and 27 (14%) domestic. The remaining 43 (22.9%) were mammals, of which 13 (6.9%) were humans, and one (0.5%) reptile. Four species (blackbird, Eurasian collared dove, house sparrow and magpie) were the origin of 81% (95/117) of blood meals coming from wild avian species (Figure 10). The other 22 blood meals came from 14 wild bird species. Analyses of feeding preference indices of the eight most abundant bird species, derived from 117 blood meal samples, indicate that blackbird and magpie were significantly preferred by *Cx. pipiens* while Eurasian collared dove was marginally preferred ($P_{blackbird}$ = 8.25, $p < 0.001$; P_{magpie} = 3.54, $p < 0.001$; $P_{collared\_dove}$ = 1.36, $p = 0.056$). Rock dove and common starling were significantly avoided (P_{rock_dove} =

0.34, p < 0.01; $P_{starling}$ = 0.089, p < 0.001). Despite their high abundance, neither common house martin nor barn swallow were detected in blood meals, suggesting that these species were significantly avoided (P_{house_martin} = 0.14, p < 0.05; $P_{barn\_swallow}$ = 0.019, p < 0.001). Finally, *Cx. pipiens* fed on house sparrow in proportion to its abundance ($P_{house\_sparrow}$ = 1.01, p > 0.05) (Figure 11). Sample size constraints prevented calculation of feeding preference indices for the other less abundant wild bird species. Domestic species were excluded as census data were unrepresentative of abundance; also, despite their relatively high occurrence in blood meals (e.g.: chicken were identified in 21 cases, 14.5% of avian species) their role in circulation of WNV is unimportant as they are not deemed competent hosts.

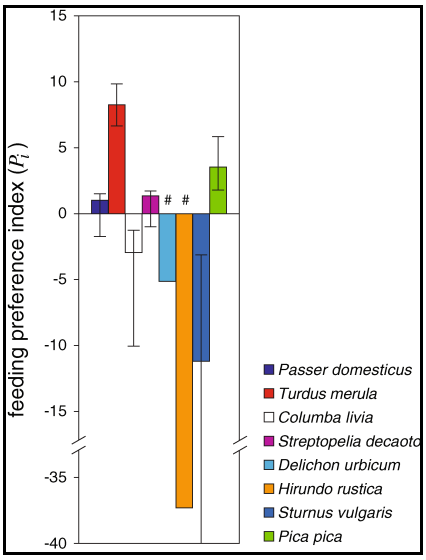

Figure 11: Feeding preference indexes (P_i) of *Cx. pipiens* mosquitoes and 95% confidence interval for the eight most abundant bird species in the Veneto region. Positive values are preferences; negative values designate avoidance and are calculated as ($-1/P_i$). Species marked '#' are calculated as conservative estimates.

For the four non-avoided species for which a sufficiently large sample size was available (see Figures 10 and 11), feeding preferences were computed distinctly in peridomestic vs. rural areas, in different seasons, or in areas with or without recorded WNV circulation. Preference for blackbird was expressed more strongly in rural than in peridomestic areas while preference for magpie exhibited the opposite pattern; for Eurasian collared dove and house sparrow, no significant differences were observed (Figure 12: n_{rural} = 53, $n_{peridomestic}$ = 64, $P_{blackbird.rural}$ = 10.97, $P_{blackbird.peridomestic}$ = 6.01, p < 0.05; $P_{magpie.rural}$ = 1.41, $P_{magpie.peridomestic}$ = 7.65, p < 0.05). Preferences for blackbird and

magpie were observed more strongly in the late than during the early part of the season, while preferences for Eurasian collared dove and house sparrow were not significantly different between the two periods (Figure 13: n_{early} = 33, n_{late} = 84, $P_{blackbird.late}$ = 25.58, $P_{blackbird.early}$ = 4.60, p < 0.001; $P_{magpie.late}$ =7.25, $P_{magpie.early}$ =1, p < 0.001). Figure 14 shows separately the seasonal change between the early and late periods for avian relative abundance (panel a) and for the proportion of blood meals (panel b). The increase in preference index for blackbird and magpie arose from differing causes: for blackbird, abundance was significantly less in the late season, but was not accompanied by a decrease in the frequency of blood meals on this species; while for magpie, the abundance remained stable but the proportion of blood meals was greater in the late season. During the latter part of the season, an increase in the number of *Cx. pipiens* bites on humans was also observed, from 2 bites (3.6% of the total blood meals) in the early season to 11 bites (8.3%) later in the season. However, sample size for bites on humans was too small, and this increase was not statistically significant. A significant preference was observed for house sparrow within sites positive for WNV (WNV+) while no preference was detected for this species in areas negative for WNV circulation (WNV-) (Figure 15: n_{WNV+} = 39, n_{WNV-} = 78 $P_{house\_sparrow.WNV+}$ = 4.04, $P_{house\_sparrow.WNV-}$ = 0.58, p <0.01). Preference for magpie was significantly higher in WNV+ areas, while the preference for blackbird was marginally higher, and feeding preference for Eurasian collared dove exhibited no significant difference between WNV+ and WNV- sites ($P_{magpie.WNV+}$ = 6.52, $P_{magpie.WNV-}$ = 1.41, p <0.01; $P_{blackbird.WNV+}$ = 14.91, $P_{blackbird.WNV-}$ = 6.97, p = 0.059).

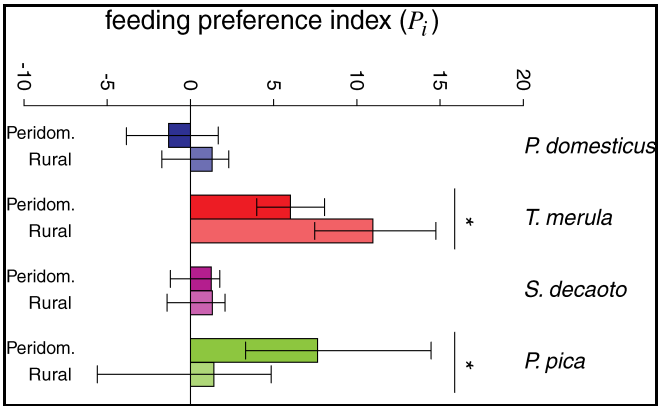

Figure 12: Spatial variation of mosquito feeding preferences between peridomestic and rural sites. Feeding preference indexes (P_i) of *Cx. pipiens* mosquitoes of the most notable bird species in Veneto in peridomestic and rural sites. Asterisks indicate statistical differences between areas (*: p < 0.05).

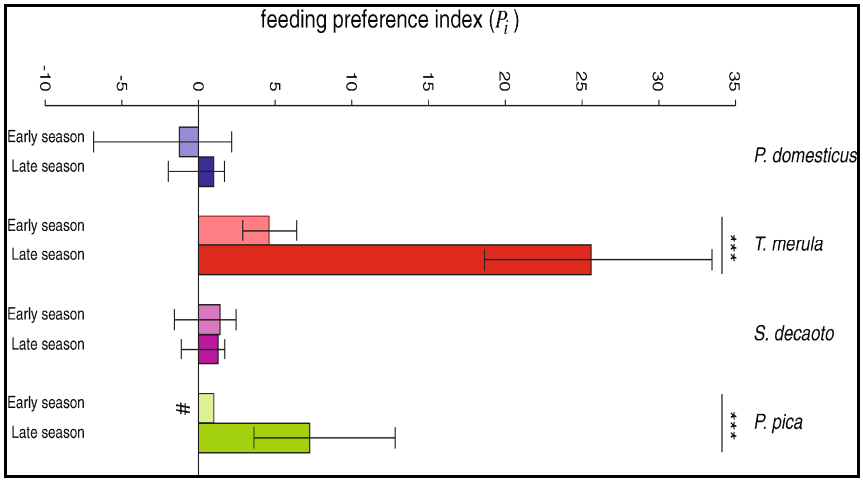

Figure 13: Temporal variation of mosquito feeding preferences during mosquito activity season. Feeding preference indixes (P_i) of *Cx. pipiens* mosquitoes of the most notable bird species in Veneto in early season (May-June period) and late season (July-September period). Columns with hash key (#) are conservative estimates. Asterisks indicate statistical differences between periods (***: $p < 0.001$).

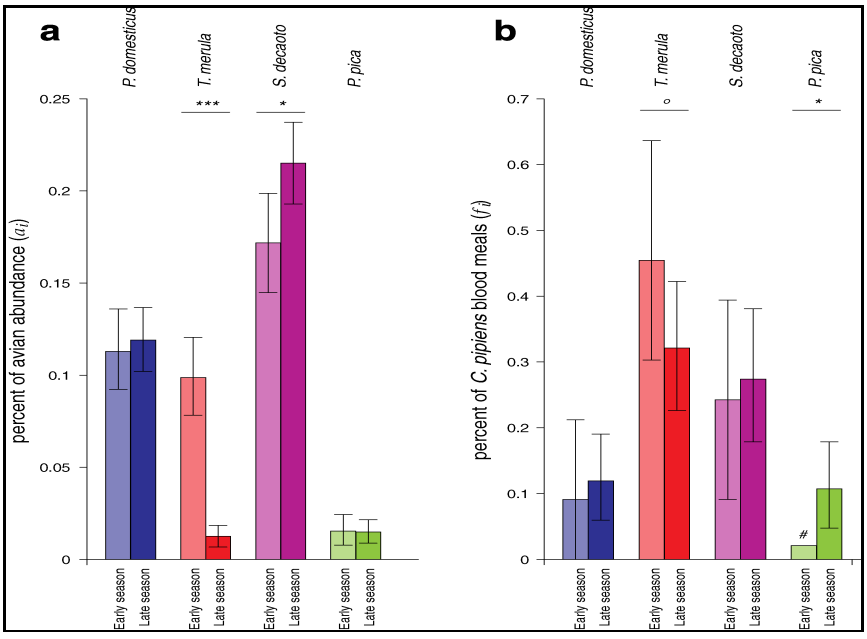

Figure 14: Temporal variation of avian abundance and blood meal origins during mosquito activity season. Percent of avian abundance (a_i) (panel a) and percent of *Cx. pipiens* blood meals (f_i) (panel b) for the most notable bird species in Veneto region. Early season: May-June period; late season: July-September period. Columns with hash key (#) are conservative estimates). Asterisks indicate statistical differences between periods (°: $p < 0.1$, *: $p < 0.05$; ***: $p < 0.001$).

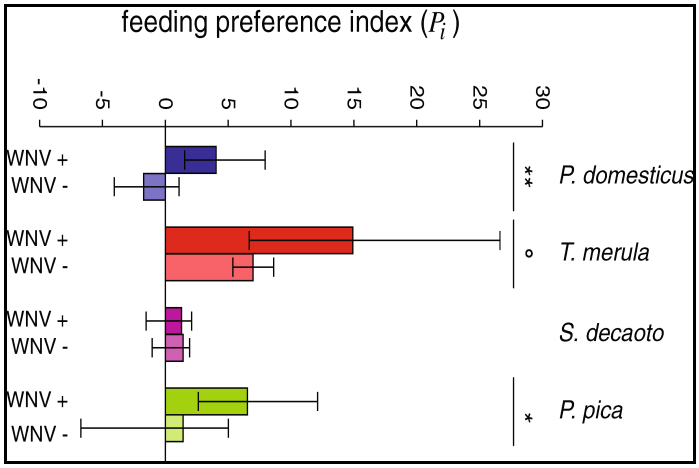

Figure 15: Spatial variation of mosquito feeding preferences between WNV positive and WNV negative sites. Differences in feeding preference indexes (P_i) of *Cx. pipiens* mosquitoes of the notable non-avoided bird species in sites where WNV circulation in mosquitoes has been observed, WNV+, or not, WNV-, in Veneto region in the 2010-2012 time span. Asterisks indicate statistical differences between periods (°: $p < 0.1$; *: $p < 0.05$; **: $p < 0.01$).

Mosquito feeding preferences in the laboratory

Odour extract solutions collected from all four bird species were attractive to *Cx. pipiens* in respect to the control (house sparrow: $\chi^2 = 4.16$, df = 1, $p < 0.05$; blackcap: $\chi^2 = 7.15$, df = 1, $p < 0.01$; blackbird: $\chi^2 = 28.88$, df = 1, $p < 0.001$; magpie: $\chi^2 = 4.33$, df = 1, $p < 0.05$) (Table 13). Comparisons among species indicated that blackbird extracts were significantly more attractive than extracts from all other species ($\chi^2 = 23.6$, df = 3, $p < 0.001$; Ryan's test, $p < 0.05$) (Table 13). Extracts from the other 3 species did not differ from each other in attractiveness (Ryan's test, $p < 0.05$) (Table 13). The preliminary trial using paired hexane / paired odour extract excluded the possibility of positional bias (hexane: $\chi^2 = 0.043$, df = 1, $p = 0.83$; blackbird extract: $\chi^2 = 0.07$, df = 1, $p = 0.79$).

Table 13: Olfactory responses of *Cx. pipiens* females to odour extracts of selected bird species. Columns description. N (%) odour: number and percentage of mosquitoes that exhibited preference to the odour extract solution; N(%) control: number and percentage of mosquitoes that exhibited preference to the solvent; χ^2 statistics: χ^2 test comparing the proportion of mosquitoes choosing odour vs. control for each classes; Ryan's test: comparison of proportions of mosquitoes choosing the odour coming from different classes (rows with the same letter indicate that proportions are not statistically different at 0.05 level).

Bird species	N (%) odour	N % control	χ^2	df	p (χ^2)	Ryan's test
Common Blackbird	39 (90.7)	4 (9.3)	28.88	1	<0.001***	A
Eurasian Blackcap	49 (66.2)	25 (33.8)	7.15	1	<0.01**	B
Eurasian Magpie	34 (65.4)	18 (34.6)	4.33	1	<0.05*	B
House sparrow	35 (64.8)	19 (35.2)	4.16	1	<0.05*	B

4.4 STUDY N.4: STUDY OF BIODIVERSITY WITH DIVERSITY INDEXES

The values of the three biodiversity indexes calculated for Veneto and Trentino, subdivided according to sampling year (2011 and 2012) and sampling area (rural and peridomestic), are showed in Tables 14 and 15.

Table 14: Biodiversity indexes values for mosquito sampling data.
NA = data not available, H' = Shannon's Index, J' = Pielou's Index, S' = Simpson's Index.

Sampling year	Region	Sampling area	H'	J'	S'
2011	Veneto	rural	NA	NA	NA
2011	Veneto	peridomestic	NA	NA	NA
2012	Veneto	rural	0,41	0,36	0,46
2012	Veneto	peridomestic	0,45	0,53	0,42
2011	Trentino	rural	0,47	0,6	0,42
2011	Trentino	peridomestic	0,26	0,37	0,66
2012	Trentino	rural	0,26	0,31	0,7
2012	Trentino	peridomestic	0,3	0,43	0,59

Table 15: Biodiversity indexes values for avian censuses data.
NA = data not available, H' = Shannon's Index, J' = Pielou's Index, S' = Simpson's Index.

Sampling year	Region	Sampling area	H'	J'	S'
2011	Veneto	rural	0,92	0,68	0,17
2011	Veneto	peridomestic	0,88	0,66	0,20
2012	Veneto	rural	0,97	0,66	0,16
2012	Veneto	peridomestic	0,89	0,74	0,16
2011	Trentino	rural	1,43	0,78	0,05
2011	Trentino	peridomestic	1,24	0,74	0,08
2012	Trentino	rural	1,45	0,79	0,05
2012	Trentino	peridomestic	1,10	0,65	0,14

5. DISCUSSION

5.1 STUDY N.1: THE ROLE OF MIGRATORY BIRDS

The transmission dynamics of flaviviruses are based on a complex relationship among virus occurrence, host and vector species community compositions (biodiversity), host behaviour, vector host preferences and competence, and environmental and climatic factors, making each spillover event a unique phenomenon resulting from the combination of all these factors (Reiter, 2010 and references therein).

Since oro-fecal shedding is an important amplification route for these viruses, assessing the rate of oro-fecal shedding in various species is important to identify the amplification chain (Komar *et al.*, 2003; Zeller & Schuffenecker, 2004; Blázquez & Sáiz, 2010; Monini *et al.*, 2010; Reiter, 2010 and references therein). Bird species differ in their susceptibility to WNV and USUV infection. For example, *Passeriformes* and *Strigiformes* are highly susceptible to USUV infection (e.g.: Savini *et al.*, 2011; Steinmetz *et al.*, 2011; Becker *et al.*, 2012; Weissenböck *et al.*, 2013) and *Passeriformes*, *Charadriiformes* and *Strigiformes* are the principal host reservoirs and amplificators of WNV, due to their long-lasting and high levels of viremia (e.g.: Semenov *et al.*, 1973; Kramer & Bernard, 2001; Reiter, 2010). Moreover, it has been suggested that a single species can act as a super-spreader of WNV (Kilpatrick *et al.*, 2006).

In previous studies, the oro-fecal shedding of USUV was detected in domestic goose *Anser anser f. domestica* (Chvala *et al.*, 2005) and chicken (Chvala *et al.*, 2006). Alternatively, 14 out of a total of 39 bird species analysed (e.g.: European greenfinch, great tit *Parus major*, European pied flycatcher *Ficedula hypoleuca*, willow warbler *Phylloscopus trochilus*, icterine warbler *Hippolais icterina*, blackcap, blackbird, European robin) previously tested negative in study also carried out in Italy (Lelli *et al.*, 2008). Moreover, shedding was also not evident in the Eurasian Jay (*Garrulus glandarius*), chicken, European nightjar (*Caprimulgus europaeus*), European bee-eater (*Merops apiaster*), barn swallow, Cetti's warbler (*Cettia cetti*), blue tit (*Parus ceruleus*) (Lelli *et al.*, 2008), and for 11 species belonging to the order *Anseriformes* tested in Finland (Lindh *et al.*, 2008).

With respect to WNV, the species tested by Lelli *et al.* (2008), the 11 species belonging to the order *Anseriformes*, screened by Lindh *et al.* (2008), and the individuals belonging to

the family *Corvidae* of British Colombia tested by Hayes *et al.* (2005) resulted negative for WNV shedding. In India, 119 species belonging to 30 families and in particular *Cuculidae*, *Motacillidae*, *Sylviidae*, *Turdidae* (order *Passeriformes*) and *Strigidae* (order *Strigiformes*) were analysed and all tested negative (Mishra *et al.*, 2012). This further corroborates the results of the current study. A study carried out in Spain did not find oral shedding in species belonging to several families, namely *Threskiornithidae* and *Accipitridae* (García-Bocanegra *et al.*, 2011). The tracheal and cloacal swabs tested in Germany were negative (Hlinak *et al.*, 2006). The tested birds belonged to order *Charadriiformes*, for e.g.: ringed plover (*Charadrius hiaticula*), little ringed plover (*Charadrius* dubius), black-headed gull (*Larus ridibundus*), some to genus *Calidris* and *Tringa*, some to the orders *Anseriformes* (*Anas* spp.), *Gruiformes* (water rail *Rallus aquaticus*, Eurasian coot *Fulica atra*) and *Passeriformes*, family *Motacillidae*, for e.g.: white wagtail (*Motacilla alba*), meadow pipit (*Anthus pratensis*) and others to the family *Corvidae* (hooded crow *Corvus corone cornix*).

On the other hand, additional studies have detected oro-fecal shedding of WNV in bird species of different families and orders. These include *Corvidae,* such as American crows (*Corvus brachyrhynchos*), fish crows (*Corvus ossifragus*), blue jay (*Cyanocitta cristata*), common ravens (*Corvus corax*), black-billed magpie (*Pica hudsonia*), little raven (*Corvus mellori*) (e.g.: Komar *et al.*, 2002, 2003; Stone *et al.*, 2005; Kipp *et al.*, 2006; Wheeler *et al.*, 2009; Bingham *et al.*, 2010); *Anatidae* (order *Anseriformes*) such as Canada goose (*Branta canadensis*), wild mallard (*Anas platyrhynchos*) and domestic goose (Swayne *et al.*, 2001; Banet-Noach *et al.*, 2003; Komar *et al.*, 2003); *Galliformes*, such as Northern bobwhite (*Colinus virginianus*), turkey (*Meleagris gallopavo*), chicken, red-legged partridge (*Alectoris rufa*) (Langevin *et al.*, 2001; Komar *et al.*, 2003; Sotelo *et al.*, 2011 and references therein); *Gruiformes*, such as American coot (*Fulica americana*) (Komar *et al.*, 2003); *Charadriiformes*, such as killdeer (*Charadrius vociferus*), ring-billed gull (*Larus delawarensis*) (Stone *et al.*, 2005); *Columbiformes*, such as mourning dove (*Zenaida macroura*) and rock dove (Komar *et al.*, 2003); *Psittaciformes*, such as monk parakeet (*Myiopsitta monachus*) and budgerigar (*Melopsittacus undulatus*) (Komar *et al.*, 2003); *Passeriformes*, such as American robin (*Turdus migratorius*), common grackle (*Quiscalus quiscula*), house finch (*Carpodacus mexicanus*), house sparrow, great-tailed grackles (*Quiscalus mexicanus*), cedar waxwing (*Bombycilla cedrorum*), northern

mockingbird (*Mimus polyglottus*), barn swallow, cliff swallow (*Petrochelidon pyrrhonota*) (Stone *et al.*, 2005; Oesterle *et al.*, 2009; Guerrero-Sánchez *et al.*, 2011); several species of diurnal and nocturnal raptors, such as Swainson's hawk (*Buteo swainsoni*), ferruginous hawk (*Buteo regalis*), peregrine falcon (*Falco peregrinus*), golden eagle (*Aquila chrysaetos*), American kestrel (*Falco sparverius*), and some species of North American owls (family *Strigidae*) like great horned owl (*Bubo virginianus*) (Komar *et al.*, 2003; Gancz *et al.*, 2004; Stone *et al.*, 2005; Nemeth *et al.*, 2007).

The results of this study further corroborate the results of a previous study also carried out in Italy, which found there was no evident oro-faecal shedding of USUV in the families *Fringillidae*, *Lanidae*, *Paridae*, *Muscicapidae*, *Sylviidae*, *Turdidae*, *Hirundinidae* and *Picidae* (Lelli *et al.*, 2008). My results also seem to suggest that birds belonging to the families *Motacillidae*, *Prunellidae*, *Emberizidae*, *Cuculidae*, *Egitalidae*, *Strigidae*, previously never screened for USUV, may not be important shedders of this virus.

Considering the migratory birds tested in Italy so far, what has been said for USUV is also valid for WNV. Moreover, this has also been confirmed in India in birds belonging to the families *Cuculidae*, *Motacillidae*, *Sylviidae*, *Turdidae* (order *Passeriformes*) and *Strigidae* (order *Strigiformes*) (Mishra *et al.*, 2012) and in Germany for *Motacillidae* (Hlinak *et al.*, 2006). Of the studies that found oro-fecal shedding for WNV, only one was carried out in Europe, but is not possible to compare it with this research mainly for two reasons: firstly, it studied a species belonging to the order *Galliformes* that was not included in the current study; and secondly, the birds were experimentally infected with the virus, and so the results may not reflect those seen in natural conditions in the wild (Sotelo *et al.*, 2011). The other studies were carried out in America and Australia and principally focused on taxonomic groups that are different from the ones that were included in this research (orders: *Columbiformes*, *Psittaciformes*, *Charadriiformes*, *Gruiformes*, *Galliformes*, *Anseriformes*, *Falconiformes*). Studies that have been carried out on *Strigiformes* and *Passeriformes*, also investigated different species to the ones included in this study (e.g.: family *Corvidae*, American robin, common grackle, house finch, house sparrow, cliff swallow, golden eagle, *Bubo* spp., *Buteo* spp., *Falco* spp.).

Accordingly, it seems that the oro-fecal shedding of USUV and WNV in *Cuculiformes* and *Piciformes* is not intense or it lasts only few days. Regarding *Strigiformes* and *Passeriformes*, their shedding seems low also for USUV, but for WNV, various families or

species could have an important role, such as *Corvidae*, *Hirundinidae*, *Icteridae*, *Turdidae*, *Fringillidae*, *Passeridae*, *Bombycillidae*, *Mimidae* and *Tytonidae*. There are several factors that could explain these different results, for example, the limited number of subjects that were tested and the taxonomical differences between the birds screened. Also, an additional reason could be the period of the year during which the study was carried out in relation to the bird's physiology: migration requires morphological and physiological changes (Hedentröm, 2008) that could interfere with the viral replication. Moreover, the oro-fecal shedding generally lasts less than 10 days (Komar *et al.*, 2003), thus being not easy to detect in clinically healthy animals as in those individuals which are migrating. Besides, the shedding is not always followed by virus transmission (e.g.: Blázquez & Sáiz, 2010; Sotelo *et al.*, 2011 and references therein).

Taking into account the need to identify the species and the timing of WNV and USUV amplification, the absence of active shedding detected in this study may also justify the absence of flavivirus infection and clinically reportable cases of spillovers events to human and animal in Trentino-Alto Adige recorded so far. Their circulation is then apparently very limited, in contrast to the high number of cases and the pathogenicity observed in animals, mosquitoes and humans in the neighbouring regions (Veneto, Lombardia, Emilia-Romagna, Friuli-Venezia Giulia: Figure 1). A possible explanation of this observed epidemiological pattern could be the low density of mosquitoes observed in this area as a result of a low habitat suitability for *Culex* spp.: a combination of low anthropization and mountainous orography of the territory, with a temperate-oceanic climate, although a sub-Mediterranean climate can be found near Lake Garda. It is not the case that most of the detections of flaviviruses monitored in this region were obtained in the territory around Lake Garda, which provides a suitable habitat for many species of mosquitoes, including *Cx. pipiens* and *Ae. albopictus* (Roiz *et al.*, 2009, 2012a). This is consistent with the observation that viruses transmitted by mosquitoes are more frequently linked to mild climate, irrigated areas, wetlands and marshes with abundant mosquito and bird populations, especially migratory birds (e.g.: Hlinak *et al.*, 2006; Calistri *et al.*, 2010b; Monaco *et al.*, 2011; Mishra *et al.*, 2012). Another co-factor to be considered is the presence of a high avian biodiversity observed in the region compared to other neighbouring regions. The relationships among high host diversity and low virus spillover have been observed in several disease models, including WNV (Kilpatrick *et al.*,

2006b; Swaddle & Calos, 2008; Ostfeld, 2009; Keesing *et al.*, 2010).

5.2 STUDY N.2: PATTERN OF FLAVIVIRUS INFECTION IN MOSQUITOES

In this study a wide distribution of AeFV in *Ae. albopictus* in Trentino and Veneto regions with variable pattern of infection was detected. For the first time, AeFV and a new sequence of an ISF were detected in *Cx. pipiens*, as well as the occurrence of USUV in Veneto region was confirmed.

The prevalence of AeFV infection in *Ae. albopictus* in both regions was higher than the prevalence of other ISFs in other mosquito species collected within the two regions, and this is in agreement with similar studies that have been carried out either in Italy (Calzolari *et al.* 2010a, 2012b; Cerutti *et al.,* 2012) or abroad (Tyler *et al.,* 2011; Machado *et al.,* 2012; Zuo *et al.,* 2014). In previous studies, seasonality has been shown to be an important factor affecting AeFV detection (Kim *et al.,* 2009; Calzolari *et al.,* 2010a). This has previously been described in Trentino by Roiz *et al.* (2012a). In this study, they detected a high AeFV prevalence (mean of 86.6%) in *Ae. albopictus*, which increased with mosquito abundance and peaked at the beginning of the season (Roiz *et al.,* 2012a). However, in the current study, seasonal variation in AeFV prevalence was not observed.

When comparing the two regions under study, in Veneto a higher viral diversity was found where three different sequences of flaviviruses were detected (AeFV, USUV and a new ISF), whilst in Trentino only AeFV was identified. Moreover, the prevalence of AeFV in *Ae. albopictus* in Trentino was higher than in Veneto, and this result is supported by previous research carried out in 2008 (Roiz *et al.,* 2009, 2012a), and it was also higher compared to the prevalence of ISFs detected in other northern Italian regions (e.g.: Lombardia, Emilia-Romagna and Piemonte: Roiz *et al.,* 2009, 2012a; Calzolari *et al.,* 2010a, 2010b, 2012a, 2013a; Cerutti *et al.,* 2012; Ravagnini *et al.*, 2012; Pautasso *et al.,* 2013). These results support the hypothesis that the local ecological and climatic conditions may shape not only the abundance and distribution of mosquito populations (Trawinski & Mackay, 2010; Roiz *et al.,* 2010, 2011) but also their viral infection pattern (Newman *et al.*, 2011; Calzolari *et al.,* 2012b; Obara-Nagoya *et al.,* 2013). In fact, the ecological and climatic conditions of the two study areas differ not only in climate but

also in the degree of anthropization, biodiversity and in land use. Veneto is a region characterized by a continental climate, high anthropization and intense agricultural and industrial activities, whilst Trentino is mostly a mountainous and forested area with a temperate climate and a lower degree of anthropization and agricultural lands. More research is now needed, however, to better understand the effect of environmental variables on AeFV ecology.

In Trentino, infection prevalence with AeFV was higher in female mosquitoes than in males in which no flaviviruses were detected, but these findings may be related to the small number of male pools analysed and to the low infection levels (Cook *et al.,* 2006; Farfan-Ale *et al.,* 2009; Roiz *et al.,* 2009, 2012a; Bolling *et al.,* 2011; Sayasombat *et al.,* 2011; Haddow *et al.,* 2013).

Regarding ISFs transmission routes, there is evidence that both vertical and horizontal transmission are possible, since trans-ovarial transmission, horizontal transmission between larvae sharing the same aquatic habitat, transmission via shared microparasites, and the infection via shared sugar-rich food sources have been reported so far (Cook *et al.,* 2013).

The phylogenetic analysis showed that the AeFV sequences detected in Veneto were very similar to those detected in Trentino, and to those previously detected in these two regions and also further afield in Italy (Roiz *et al.,* 2009, 2012a; Calzolari *et al.,* 2012b). The high sequence identity of the virus that was detected in *Ae. albopictus* mosquitoes to those isolated in Japan would corroborate the hypothesis that these two viruses are the same or closely related viruses (Roiz *et al.*, 2012a), but the analysis of the complete genome of these strains would be necessary to confirm it. It is thought that *Ae. albopictus* has most likely brought AeFV during its recent expansion from Japan to North America and Europe (Hawley *et al.,* 1987; Rai, 1991; Benedict *et al.,* 2007; Enserink, 2008; Bonizzoni *et al.,* 2013). It has also been suggested that the nucleotide sequence is relatively well-conserved because ISFs are not harmful to the mosquito host and because NS5 is not affected by host immunity (Obara-Nagoya *et al.*, 2013). This result agrees with the discovery of high genome similarity between ISFs circulating in several European countries demonstrating the widespread presence of different ISFs in Europe related to other isolated or detected worldwide and the high rate of gene flow among mosquito populations (Calzolari *et al.,* 2012b; Cook *et al.,* 2012; Obara-Nagoya *et al.,* 2013).

This study reports for the first time the detection of AeFV sequences from *Cx. pipiens* from Veneto and Trentino. This result was unexpected because, at least to my knowledge, ISFs were thought to be maintained principally in a specific host genus: for example, AeFV in *Aedes* spp., OcFV in *Ochlerotatus* spp. and CxFV in *Culex* spp. (Sánchez-Seco *et al.,* 2010; Calzolari *et al.,* 2012b; Obara-Nagoya *et al.,* 2013). This close association between viral strains and mosquito genus has also been found in other studies carried out in northern Italy (Calzolari *et al.,* 2010a; Cerutti *et al.,* 2012; Roiz *et al.,* 2009, 2012a). Further research efforts are therefore required to clarify the ecological rules that drive transmission and circulation of these viruses among different genus and how cross-infection could lead to potential biological advantages for the viruses other than affecting the vectorial capacity of mosquitoes.

In one pool of *Cx. pipiens* collected in Veneto was also found a sequence of a new ISF that has not been previously reported. Although further work is needed to confirm the identity of this new species of virus, this finding demonstrates that potentially new flaviviruses could be detected in the near future in these species of mosquito. Furthermore, it is unclear so far if AeFV and/or these new ISFs in *Cx. pipiens* (main vector of WNV and USUV in Italy) may interact with other pathogenic flaviviruses within the vector, thus affecting, either in positive or negative direction, their transmission dynamics. More studies on viral interference of these ISFs with other pathogenic flaviviruses in vitro (cell culture) or in vivo (mosquito inoculation) are therefore urgently required to test this hypothesis.

The detection of USUV in Veneto during two consecutive years showing a total sequence homology in 1000 nt with the USUV strain previously detected in the neighbouring region (Emilia Romagna) in 2009 suggests a wide endemic viral circulation of this strain in northern Italy and in this specific area.

The viral isolation failed for the new ISFs and for AeFV was successful only from four pools of mosquitoes out of 23. This is not surprising since other studies have also reported problems in isolating ISFs in cell cultures and the presence of an evident CPE sometimes appeared to be viral-strain specific (Hoshino *et al.,* 2009; Kim *et al.,* 2009; Calzolari *et al.,* 2010a; 2012b; Huhtamo *et al.,* 2012; Chen *et al.*, 2013).

Finally, for the first time to my knowledge, the new invasive specie *Ae. koreicus* has been screened for *Flavivirus*, and all the samples tested were negative. However, since

only a very small number of mosquitoes was analysed this time (n = 11 females), further studies are needed to verify the apparent lack of ISFs' infection in this mosquito species.

5.3 STUDY N.3: ROLE OF THE FEEDING PREFERENCE OF MOSQUITOES

Assessing the feeding preference of *Cx. pipiens* including its biotypes is pivotal to understand and model the temporal and spatial dynamics of emerging flaviviral infection in Europe (Dye & Hasibeder 1986; Bolzoni *et al.*, 2015).

Analysing fresh fully engorged females is essential to increase the success of host identification. Previous studies have shown that the degree of blood meal digestion status of fed mosquitoes can alter the host composition identified in blood meal analysis (Thiemann & Reisen, 2012; Thiemann *et al.*, 2012). However, in Thiemann & Reisen (2012) and Thiemann *et al.* (2012) mosquito sampling procedures were different in respect to this study; in particular, this research used BG traps to collect engorged mosquitoes, while in those study CO_2-CDC traps and gravid traps were used. In a recent study (Roiz *et al.*, 2012c) it has been observed that the number of freshly engorged mosquitoes collected with BG traps is higher than using CDC traps. The digestion status of mosquito blood meals was visually scored by using the Sella score ordinal rating system *S* (Martinez-de la Puente *et al.*, 2013) only for mosquitoes collected in Trentino. Martínez-de la Puente *et al.* (2013) showed that Sella score, a measure of the degree of blood meal digestion status, significantly affects the success of blood meal identification, with a significant drop in success of host identification for mosquitoes containing a blood meal in an advanced stage of digestion (Sella score higher than 5). Following the outcome of digestion evaluation of Trentino samples and considering that mosquito collection protocol used in Veneto was identic to that followed in Trentino, although quantitative data on the status of the blood meals of Veneto were not collected, it is possible to affirm that most of the blood meals identified at host species level derived from fresh fully engorged females.

Previous studies have indicated house sparrow and blackbird as preferred host species and there is still a debate on the feeding behaviour of hybrid and *f. molestus* biotypes with its implications of eco-epidemiology of zoonotic viral infections (Roiz *et al.*, 2012b;

Gomes *et al.*, 2013; Rizzoli *et al.*, 2015a, 2015b). In Trentino, based on the results obtained from field-collected data, the analysed *Cx. pipiens* population, characterized by a large prevalence of the *f. pipiens* biotype, resulted to have principally ornithophilic habits but also to feed on humans, supporting the results from previous studies (Muñoz *et al.*, 2012; Gomes *et al.*, 2013; Osório *et al.*, 2013). Intriguingly, also considering the CQ11 limit to identify the biotypes at the individual level, the fraction of *Cx. pipiens* population identified as *f. molestus* and hybrid biotypes (about 15%) seems to have a marked preference for birds, confirming what found in Portugal by Gomes *et al.* (2013). This result has been further corroborated by the results obtained in the behavioural bioassays showing a clear ornithophilic preference of the *Cx. pipiens* lab-colony tested and mainly composed by *f. molestus* biotype and suggesting that this biotype can play an important role as main bridge vector favouring the spillover of the viruses from the birds reservoir hosts to human, especially in highly anthropised conditions (Marcantonio *et al.*, 2015).

The computation of feeding preference index indicated the Eurasian tree sparrow was a preferred host in Trentino, but this preference may be overestimated because of variation in local abundance and/or low relative abundance of this species in the avian community. Therefore, the absolute contribution of the Eurasian tree sparrow in terms of *Cx. pipiens s.l.* feeding preference could be less significant with respect to house sparrow and blackbird contributions.

Instead, field data indicated blackbirds were clearly a preferred species for the *Cx. pipiens* population in Trentino. Although less abundant than house sparrow, blackbird is responsable for a higher number of blood meals, confirming what found by Roiz *et al.* (2012b) in the same field study area and by different studies in other European regions (Muñoz et *al.*, 2012; Gomes *et al.,* 2013; Rizzoli *et al.*, 2015a). Similarly, in Veneto, blackbird and house sparrow, other than magpie and Eurasian collared dove, resulted as the species most frequently bitten by *Cx. pipiens*. This result confirms previous studies on blood meal analysis conducted in European countries showing that *Cx. pipiens* fed most frequently on birds belonging to order Passeriformes, and in particular on house sparrow and blackbird (Muñoz *et al.*, 2012; Roiz *et al.*, 2012b; Gomes *et al.*, 2013). Intriguingly, in both regions blackbird resulted significantly preferred and house sparrow fed upon opportunistically. Together, these findings suggest that blackbird (as preferred species) in

particular, along with house sparrow (as abundant species that are opportunistically fed upon) and, in Veneto also magpie and Eurasian collared dove, have the potential to play a crucial role in the circulation and amplification of WNV in Italy.

Unfortunately, the reservoir role of the blackbird for WNV in Europe is still unknown, but blackbird represents a major host for other viruses transmitted by *Culex* mosquitoes which are closely related to WNV, such as USUV and Sindbis virus (Lundstrom *et al.*, 2001). The importance of blackbird in northern Italy therefore, mirrors the importance of the American robin (*Turdus migratorius*) in the United States (Kilpatrick, 2006a, 2006b; Hamer *et al.*, 2011; Simpson *et al.*, 2012; Janousek *et al.*, 2014), and suggests that the true thrushes of the genus *Turdus* may play a key role in the transmission of zoonotic pathogens transmitted by *Culex* mosquitoes.

Weaver & Reisen (2010) reported that the introduction of the house sparrow has contributed to the emergence of WNV in the Nearctic region. They also suggested that, given its widespread presence, invasiveness and high host competence for most of the WNV strains (Del Amo *et al.*, 2014a, 2014b and references therein), it may represents a maintenance and amplification host also in Europe. However, the reservoir competence of this species for the European WNV strains is still poorly known, although variation in host competence for the different circulating strains has been recently observed (Del Amo *et al.*, 2014a, 2014b and references therein). Since house sparrow and blackbird are frequent visitors of human settlements (like houses, gardens, agricultural areas, and urban parks), and given the ornithophilic or catholic feeding habits of hybrids and *f. molestus* biotypes emerged from this and previous studies, the proximity of these two bird species to anthropic environment, may increase the possibility of accidental transmission of WNV to humans.

In Trentino the preference for blackbird observed in field trials with a *f. pipiens*-prevalent population were confirmed, through laboratory behavioural assays, in a *f. molestus*-prevalent colony, suggesting that the genetic, physiological and behavioural differences between biotypes do not affect this aspect of *Cx. pipens* feeding habits. Similarly, the preference of *Cx. pipiens* for the blackbird in Veneto was confirmed by both methods, suggesting that the high feeding preference index is the result of intrinsic mosquito preference. On the other hand, the behavioural bioassays did not confirm preferences for magpie in respect to other species, suggesting that the observed feeding

preference index in the field strongly depends on host ecology/behaviour of this species in this region. Behavioural bioassays in laboratory identify intrinsic preferences, since they exclude potentially confounding variables such as environmental conditions, bird abundance and behaviour. Moreover, the results obtained in both regions under study indicate that studying the role of different hosts considering only the blood meal analysis (host feeding habits) may provide misleading information. This supports the conclusion that the overall abundance of avian species is likely to be a poor indicator of importance in disease transmission, as has been demonstrated in the USA (Hassan *et al.*, 2003; Kilpatrick *et al.*, 2006b; Hamer *et al.*, 2011). It is important to couple host feeding habits with data of host relative abundances, coming from the census of the avian community living around the mosquitoes sampling sites.

Overall these outcomes suggest that while mosquito feeding behaviour in the field can be partially ascribed to intrinsic feeding preferences, it is a plastic pattern which can be overridden by environmental circumstances such as avian abundance or behaviour (Takken & Verhulst, 2013). For instance, the observed avoidance of the barn swallow and the common house martin can be explained by their behaviour: both are insect-eating birds that feed on the wing, and are largely inaccessible to feeding mosquitoes for a significant fraction of the day (Turner & Rose, 1989). Differences in mosquito preference between blackbird and common starling, both of which feed on or near the ground, can perhaps be partly explained by the crepuscular foraging habits of the former, which fits with *Culex* mosquito feeding habits (Farajollahi *et al.*, 2011; Gray *et al.*, 2011), and the diurnal feeding habits of the latter (Snow & Perrins, 1998). Similarly, differences in mosquito preference between blackbird and house sparrow, could be partially justified with blackbird usually searching food more often on the ground and sleeping in bushes while house sparrow lays more often on trees and house roofs (Mullarney *et al.*, 2013). Recent studies carried out in North America demonstrated that *Culex* mosquitoes feed more actively on species roosting at high altitude (such as American robin) rather than at the lower altitude, so that variation in habitat use by host and vectors and social aggregation by hosts influence vector-host interaction (Janousek *et al.*, 2014). The highly variable nature of mosquito feeding preference suggests that broader inferences about the significance of blackbird, or genus *Turdus* in general, must be made cautiously: similar studies in Europe should be carried out in other areas and habitats.

In Veneto and Trentino, only a part of the blackbird breeding population present in agricultural and urban areas is resident. After breeding (March to July), many juveniles and adults move from nesting areas to sites rich in fruiting plants (e.g.: *Sambucus nigra*, *Viburnum lantana*, *Cornus sanguinea*, *Prunus spinosa*, *Prunus padus*) where they moult and accumulate fat reserves prior to the autumnal migration (Snow & Snow, 1988; Berthold, 2001). Because of these movements, only a relatively small number are available as potential hosts for *Cx. pipiens* mosquitoes. On the other hand, house sparrow, magpie and Eurasian collared dove are resident, but the density of their populations increases at the end of the summer because newly born juveniles add to the adult populations (Snow & Perrins, 1998). Although a preference for blackbird was consistent within the current study (among sites, seasons, and methods), further analyses of data available in Veneto showed that the degree of preference for blackbird and for other species shifted both seasonally, and with habitat. Actually, a sharp decline in the availability of blackbirds late in summer during the mosquito activity season (i.e. July-September) was reflected by a decrease in blackbird blood meals. However, when abundance is taken into account it is apparent that the decrease in blood meals is less than would be expected, as revealed by an increased preference index. At the same time, we observed a sharp increase in feeding on magpies. Our analyses suggest that the overall apparent preference for magpies is entirely driven by the late season preference. The observed increase in magpie feeding preference is likely to be driven in part by the decreased availability in blackbirds, but also by the increase in communal roosting in late summer/early fall that follows the end of the magpie breeding season (Georgiev & Iliev, 2009). On the other hand, blackbirds maintain their home range throughout the year even if during winter some latitudinal migration weather dependent may occur. Clustering around winter food resources might occasionally occur but the species, in the study area, does not properly roost or nest in colonies (Cramp, 1988). This interpretation is in agreement with others (Farajollahi *et al.*, 2011) highlighting that, for nocturnal or crepuscular feeding vectors as *Cx. pipiens*, the over-utilization of a host species can arise from an overlap between mosquito microclimate selection and host roosting behaviour. Human cases of WNV in Northern Italy tend to peak in August-September (e.g.: ECDC, 2013). This seasonality may reflect the variation in feeding preference by mosquitoes, as observed in the USA where a rise in human WNV infections coincides with a shift in

feeding behaviour following the dispersal of the American robin (Kilpatrick *et al.*, 2006a). Further studies conducted in Alabama (southern USA) showed that host phenology and winter temperatures may also contribute to the temporal shift in mosquito feeding pattern (Burkett-Cadena *et al.*, 2012).

Regarding the possible factors that can explain the different attraction of mosquitoes towards different bird species, several hypothesis can be suggested.

A possible explanation is the difference in the composition of odour bouquet emitted by each bird species (Campagna *et al.*, 2012). Unfortunately, there is still a lack of knowledge of the chemical composition of body odours of many European bird species. It could be interesting in future to repeat the behavioural bioassay using synthetic volatiles identified from the headspace extracts of a larger number of local birds species. The chemical analysis of their headspace extract solutions and subsequent electrophysiological recordings could help in selecting the single volatile compounds involved in host recognition and in evaluating their activity even at longer range in either semi-field or field conditions (Syed & Leal, 2009).

Another explanation could be the difference in body mass, since larger hosts would release a higher quantity of volatile molecules because of their broader body surface and higher carbon dioxide and heat production (Takken & Verhulst, 2013). Moreover, a bird species could be more or less exposed to mosquito bites because of its behaviour and the habitat it attends, as already discussed above.

Regarding the effect of age and sex on the feeding preference, for the *Cx. pipiens s.l.* tested in Trentino, both females and juveniles blackbird were found to be less attractive than adult males. Changes of the chemical composition of the volatile compounds or of the quantity of body secretions in relation to age, sex, social and/or reproductive behaviours have been reported in other species (Campagna *et al.*, 2012). Captures of wild birds for the laboratory experiment were carried out during the second half of the breeding season, which is characterized by a peculiar hormonal pattern that influences gland secretions (Campagna *et al.*, 2012). Consequently, the differences in attractiveness found within different categories of blackbird are more probably due to differences in the chemical composition of the body odour rather than to the body size, since body sizes of these categories are very similar. Regarding the influence of age, some studies indicated that mosquitoes are more attracted to adults and older nestlings than to

hatchlings, others found no differences and others reported that nestlings are preferentially selected (Burkett-Cadena *et al.*, 2010 and references therein). Nestling and juvenile birds have also been proposed as important reservoirs for arboviruses, potentially increasing human infection risk (Pérez-Ramírez *et al.*, 2014).

With respect to the differences in feeding preference in relation to environmental conditions, in Veneto mosquito feeding indices reflect a stronger preference for blackbirds in rural areas, and for magpies in peridomestic environments. The preference for magpies in peridomestic areas, where the contact rate with humans is higher, suggests that they may be an important bridgehost for WNV transmission to humans. Data from the WNV surveillance program carried out on sinantropic corvids in the WNV circulation area of northern Italy has evidenced that magpies contribute 70% of the WNV positivity in corvids, suggesting a significant role for magpies in WNV transmission (IZSAM, 2015). In addition, the potential importance of magpies in WNV amplification and transmission could be supported by the observations that the magpie feeding index is significantly greater in areas with known WNV circulation, compared to sites where WNV has never been detected. However, this hypothesis needs to be validated. Since WNV positive and negative sites in the study area were spatially clustered, alternative hypotheses (such as different patterns in avian community, habitat, and climatic conditions) may explain the observed differences in WNV circulation.

Blackbirds and magpies have been found infected or at least exposed (seroconversion) to WNV in Europe on several occasions (Buckley *et al.*, 2003; López *et al.*, 2008; Balança *et al.*, 2009; Lelli *et al.*, 2012; Valiakos *et al.*, 2012). However, studies on their competence for the strain of WNV circulating in Europe are still very limited. Experimental studies on house sparrow both in USA and in Europe showed that this species may develop high levels of viremia. However, competence may differ depending on the virus strain tested, and host competence can vary geographically (Del Amo *et al.*, 2014a; Pérez-Ramírez *et al.*, 2014). Species belonging to genera *Turdus* and *Pica* are highly competent hosts for the WNV strain circulating in North America (Komar *et al.*, 2003; Pérez-Ramírez *et al.*, 2014), but unfortunately no studies have been performed on these species so far with European WNV strains. For these reasons, estimates of host competence obtained in different epidemiological contexts must be treated with caution.

5.4 STUDY N.4: STUDY OF BIODIVERSITY WITH DIVERSITY INDEXES

Analysing the results obtained from the calculation of biodiversity indexes some interesting differences become evident, which confirm what emerge looking at the field data reported in Tables 2, 6, 7 11 and 12, and corroborates what resulted in the other part of this research.

Regarding mosquito sampling data, values obtained from Shannon's Index (H') clearly show that in Veneto the number of mosquito species and the number of individuals of each species are higher than those registered in Trentino. This result can be explained taking into account the difference in climate, land use and orography existing between the two regions, as already discussed above.

Moreover, the relative abundance of mosquito species living in Veneto is more similar than that registered in Trentino, both in rural and peridomestic areas (see Simpson's - S' and Pielou's - J' Indexes). This is evident also looking at the data reported in Table 2. However, this homogeneity was present also in Trentino in rural area of 2011 but has decreased during the following year. The reason of this is probably the increase of the relative abundance of *Ae. albopictus* species in both type of sampling areas.

Comparing Trentino and Veneto data of 2012, it is evident that in both regions the diversity of mosquito species and their abundance are higher in peridomestic than in rural areas (see H'). Probably the peridomestic environment, having more green and uncultivated areas and free waters, fits better with the biological needs of mosquitoes in terms of feeding host, food supplies, shelters and breeding and ovideposition sites. Conversely, the dominance of few mosquito species is higher in rural than in peridomestic areas (see S' and J').

Regarding bird census data, values obtained from H' show that in Veneto the number of bird species and the number of individuals of each species are lower than those registered in Trentino, in both years of study and sampling areas. This result confirms what already told above about the fauna living in the two regions and can be also explained considering that the broader wild and uncultivated surface in Trentino than in Veneto represent a better environment to satisfy bird needs. For the same reasons, both in Trentino and Veneto, in 2011 and 2012, the diversity of bird species and their abundance are higher in rural than in peridomestic areas (see H'). Taking into account

the relative abundance of each species, the values of S’ and J’ obtained suggest that generally the bird species forming the communities censused are balanced, without any species significantly outnumbering the others. However, these indexes in both years and areas are higher in Veneto than in Trentino, probably because of the relatively higher abundance recorded in Veneto for rock pigeon, house sparrow, Eurasian collared dove and common starling.

6. CONCLUSIONS

Flaviviruses, in particular WNV and USUV, are spreading in Europe and although the number of human cases is still sporadic, it is fundamental to understand the ecological mechanism driving their emergence and spread. Despite the limitations discussed above, this research provides new and valuable insights into the eco-epidemiology of flaviviruses in two regions of the north-east Italy, through the study of the possible role played by the wildlife with particular respect to bird species and *Cx. pipiens* mosquitoes.

In this study I did not identify active oro-fecal shedding of WNV and USUV in 322 individual birds belonging to 18 transaharian and 21 intrapaleartic species. The lack of detection of active virus shedding in these species, however, does not exclude the circulation of these viruses within the region of Trentino, as noted in a previous study (Rizzoli *et al.*, 2007). Considering the high rate of animals and goods movements into this territory, and possible future climatic changes, the temporal and spatial dynamics of pathogens, vectors and avian hosts could also change (Fuller *et al.*, 2012); therefore, the circulation of flaviviruses in Trentino and Veneto needs to be carefully monitored in the future.

Since the composition and the structure of a living community can heavily influence the circulation of certain virus and ultimately also the disease risk for animals and humans, biodiversity indexes are a useful mathematical tool to study the composition of a community and help to discover differences in term of space and time, supporting and corroborating what can be found following other approaches. In this study, the diversity of biodiversity indexes values obtained support the hypothesis that part of the difference in the flavivirus ecoepidemiology existing between Trentino and Veneto can be due to difference in mosquito and bird communities.

The viral screening of mosquitoes collected within entomological surveillance programs has been demonstrated to be an effective early warning system for the detection of viral circulation and for predicting the infection hazard for human and animal (Calzolari *et al.*, 2013b). This study highlights the urgent needs for more experimental studies and research in the field of viral co-infections in mosquitoes vectors to clarify the consequences of high prevalence of ISFs in *Ae. albopictus* and *Cx. pipiens* on the mosquitoes vectorial capacity.

Although further molecular investigation using microsatellite-based approaches will be needed to accurately determine the intra-specific composition of *Cx. pipiens*, this research contributes to a better insight into the ecology and feeding behaviour of this species providing evidence of its host selection toward a specific fraction of the avian host population. These findings are crucial in order to implement targeted eco-epidemiological research and surveillance. However, to better understand the role of the biotypes of this species in the ecology of WNV and other emerging flaviviruses, to further model the risk for WNV transmission in relation to the local host community composition and abundance, and to set up focused surveillance plans, reservoir competence study on synanthropic bird species in Europe are needed.

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