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First detection of replicative deformed wing virus (DWV) in *Vespa velutina nigrithorax*

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Abstract

In order to investigate the possible infection of deformed wing virus (DWV) in the Asian hornet *Vespa velutina nigrithorax* du Buysson, in 2017 hornet workers were sampled during their predatory activity in April/May (early-season) and July (mid-season), while newly-emerged males and gynes were sampled in November (late-season). By strand specific RT-PCR replicative DWV was detected in workers sampled in July and in the newly-emerged specimens collected in November, proving that DWV can infect the Asian hornet. Sequence analysis of partial DWV genome indicates that the virus belongs to the worldwide diffused and less virulent genetic variant, DWV type A. This is the first report of DWV infection in *Vespa velutina* Lepeletier. This result suggests a possible role of this RNA virus in a natural re-equilibrium of the relationship between the prey (honey bee) and the predator (Asian hornet).

Key words: *Vespa velutina*, Asian hornet, deformed wing virus, honey bees, strand specific RT-PCR.

Introduction

Asian hornet *Vespa velutina* Lepeletier (Hymenoptera Vespidae), commonly known as "yellow-legged hornet", is a honey bee predator native of South East Asia (Monceau *et al*., 2014). The hornet predatory activity is achieved by hovering in front of the entrance of honey bee hives (Abrol, 1994; Monceau *et al*., 2013a). Regarding prey/predator relationship, *Apis cerana* F. has developed a complex signal and behaviour mechanisms to protect itself and the hive from hornet attacks due to the prey/predator co-evolution, such as endothermic heat production or heat-balling (Ken *et al*., 2005; Tan *et al*., 2010; 2012a; 2012b).

In 2004, *Vespa velutina nigrithorax* du Buysson reached South West France (Aquitaine region) probably by passive transport (Villemant *et al*., 2006). Since then, the Asian hornet rapidly spread in French territory reaching Spain in 2010, Portugal in 2012, Italy in 2013, Germany in 2014 and more recently Belgium, Switzerland and United Kingdom (López *et al*., 2011; Grosso-Silva and Maia, 2012; Rome *et al*., 2013; Demichelis *et al*., 2014; Witt, 2015; Bertolino *et al*., 2016; Budge *et al*., 2017). In Italy it was confined in Liguria and Piedmont regions since 2016, but in the last two years has also been identified in other Italian regions, Veneto, Lombardy and Tuscany (Bortolotti *et al*., 2016; www.stopvelutina.it).

In Europe, the yellow-legged hornet predatory behaviour towards honey bees, varies with its life cycle and with the flying activity of honey bees in front of the apiaries (Monceau *et al.*, 2013a; 2013b). The predatory pressure increases from spring to summer when the hornet colony has reached the maximum population density (Monceau *et al.*, 2013a; 2013b).

In the countries of origin, *V. velutina* produces monogynous annual colonies. The biology and life cycle of *V. velutina nigrithorax* in the areas of recent introduction in Italy has not been described yet. The available data come from the French regions, which anyway could show some discrepancies compared to the Italian situation, given that Liguria has a Mediterranean climate more than a continental one. According to Monceau and colleagues, the founder queen leaves the diapause between the end of February and the beginning of March, and immediately starts to build the embryo (or primary) nest (Monceau *et al.*, 2014). The first workers emerge at the beginning of May and start the predation in front of the hives between June and July. During this period the colony increases exponentially and in most of the cases the nest is moved to a new location (secondary nest). At the end of summer, the colony reaches its largest size and starts the production of gynes and males, which goes on from mid-September to end November (Monceau *et al.*, 2014).

The reports collected in Liguria region during the last three years (www.stopvelutina.it) indicate a period of queen emergence and nest foundation in agreement with the above described life cycle. The first queens flying around were seen between the half of February and the beginning of March and the primary nests with founder queen were observed around the half of April. The presence of workers in the primary nests and in front of the hive occurs from end of April to end of May. Is this interval of time starts the predation activity of workers in front of apiaries that increase greatly from the half of June. The large secondary nests were reported starting from the half of July and during the whole summer and autumn, and in some cases the nests were reported to be inhabited by adult wasps until the end of November.

Due to *V. velutina* predation, the *Apis mellifera* L. foraging activity is inhibited, resulting in colonies depopulation and increase of colony death rate during winter (Monceau *et al*., 2013a; 2013b).

The success of invasive exotic species is usually attributed to the lack of specialist enemies (Keane and Crawley, 2002) and then introduce the importance to find a specific pathogen for *V. velutina.*

Honey bees are exposed to several pathogens. Among them an important role is played by deformed wing virus (DWV), a ssRNA(+) virus belonging to the *Picornaviridae* family within the *Iflavirus* genus (de Miranda and Genersch, 2010; Genersch and Aubert, 2010).

DWV has been grouped in three genetic variants defined as type A, B and C (McMahon *et al*., 2016; Mordecai *et al*., 2016b). DWV type A is responsible for asymptomatic or symptomatic honey bee infection as recently proven using purified virus of pure recombinant DWV RNA (Lamp *et al*., 2016). The latter is characterized by deformed or missing wings, shortened abdomens and premature death leading ultimately to the collapse of the bee colony (de Miranda and Genersch, 2010). The virus is distributed worldwide and it is transmitted to honey bees mainly by the bite of the ectoparasitic mite *Varroa destructor* Anderson et Trueman. Within a bee hive, DWV can be transmitted also through direct contact especially in case of high level of infection (Ball and Allen, 1988; Nordström, 2003; Shen *et al*., 2005; Lanzi *et al*., 2006; Gisder *et al*., 2009; Francis *et al*., 2013; Giusti *et al*., 2016;). Recent studies have proven the presence of DWV on flower pollen, pollen load and in other bee products, supporting other possible horizontal transmission routes of the pathogen (Chen *et al*., 2006; Yue *et al*., 2007; Mockel *et al*., 2011; Mazzei *et al*., 2014). DWV titre in honey bee varies according to the season with the lowest in winter and the highest in late summer/early autumn (Porrini *et al*., 2016).

DWV has been recently identified in symptomatic and asymptomatic form in *Vespa crabro* L., a native predator of *A. mellifera*, suggesting the possibility that the virus can be transmitted to other Vespoidea, such as *V. velutina,* by eating infected honey bee foragers (Forzan *et al*., 2017b).

The aim of this investigation was to evaluate the presence of DWV in *V. velutina nigrithorax* specimens collected in Italy during 2017, to reveal a possible virus spill over from *A. mellifera* to the Asian hornet.

Materials and methods

Sampling

During 2017 in Liguria region (Italy), fifteen *V. velutina nigrithorax* workers were sampled in the early-season (23rd April - 30th May) in Airole area (43°52'26.8"N 7°33'00.3"E), fifteen in the mid-season (24th July) in Bordighera (43°46'45.2"N 7°39'50.3"E), Sanremo (43°49'24.8"N 7°44'24.2"E) and Dolceacqua (43°51'25.5"N 7°37'20.3"E) areas and three gynes and three males were collected in the late-season (15th November) in Ventimiglia within the botanic garden "Giardini Hanbury" (43°46'57.9"N 7°33'14.7"E). The early-season and mid-season samplings were performed by catching the hornets in front of the apiaries during their predatory activities.

The late-season sampling was performed collecting six newly-emerged specimens all belonging to the same single nest.

The hornet wet weight measure was used in order to determine the caste of the three newly-emerged females collected in late-season (Rome *et al*., 2015).

Total RNA extraction

The total RNA extraction of the early-, mid- and late-season sample was performed differentially.

Early- and mid-season RNA extraction was performed from three pools each (five hornet's specimens per pool) so that a three sample replicates per season was obtained.

Due to the lack of specimens (three males and three gynes), the RNA extraction of the late-season sample was performed using a single specimen, for a total of six RNA extractions. Each specimen was considered as a replicate (three replicates for the males and three for gynes).

In order to perform an internal experiment control, each late-season specimen has been cut longitudinally to guarantee part of the precious samples in case of RNA extraction failure (figure 1).

Total RNA was extracted by RNeasy Mini Kit (Qiagen, Hilden, Germany) following tissue homogenization using a TissueLyser II (Qiagen) carried for 3 minutes at 25 Hz. Samples were eluted in 30 µl RNase-free water, quantified on Qubit by using the RNA HS assay kit (Life-Technologies, Stafford, USA) and stored in aliquots at −80 °C until use. As a negative control, RNA obtained from blood-feeding mosquito (*Culex pipiens* L., Diptera Culicidae), collected in the same area, was extracted.

RT-qPCR

A one step assay for absolute quantification of DWV RNA (RT-qPCR) was performed by Quantitect Probe RT PCR Kit (Qiagen) using a Rotorgene Corbett 6000 (Corbett Research, Australia) as previously described (Mazzei *et al*., 2014). All reactions were carried out in duplicate using a dilution series of standard DWV RNA ranging from 2×10^5 to 2 copies/µl.

Figure 1. Longitudinal section of a newly-emerged male of *V. velutina nigrithorax*.
(In colour at www.bulletinofinsectology.org)

Strand-specific RT-PCR

The specific detection of positive and negative strand DWV RNA was performed by a two-step RT-PCR as described by Mazzei and colleagues (Mazzei *et al*., 2014). Reactions were performed for all RNAs extracted using QuantiTect Reverse Transcription Kit (Qiagen), with specific primer Fw 8450: 5'-TGGCATGCCTTGTTCACCGT-3' (nt. 8450-8469) or Rev 8953: 5'-CGTGCAGCTCGATAGGATGCCA-3' (nt. 8953-8932).

A semi-nested PCR was carried out with the RT-qPCR DWV primer Fw: 5'-TTTGACATTGAGCTACAAGACTCG-3' (nt. 8685-8708) and the primer Rev 8953, to generate a 268 bp fragment. PCR products were analysed on a 1.5% agarose gel. The nucleotide positions cited throughout the text refer to the DWV reference sequence (NC_004830.2).

Sample sequencing and phylogenetic analysis

Strand specific RT-PCR positive samples were sequenced (BMR Genomics, Padova) and analysed using BioEdit Software (Hall, 1999). The sequence of DWV-A, DWV-B and DWV-C types (accession numbers NC_004830.2, NC_006494.1 and CEND01000001) were aligned with the partial sequence of our samples obtained from *V. velutina nigrithorax*. This data was further supported by phylogenetic analysis performed to compare our DWV sequence isolated from *V. velutina* (504 bp) to other viral sequences from *A. mellifera* available on GenBank. Sequence homology to DWV type A, B and C was calculated by p-distance (Tamura *et al.*, 2013).

Results

Caste determination of late-season newly-emerged females

The newly-emerged females wet weight resulted 420 mg, 487 mg and 517 mg.

The wet weight of each late-season sampled gyne is heavier than the 386.4 mg recorded as highest wet weight in *V. velutina* workers collected in November (Rome *et al.*, 2015).

Detection of DWV in *V. velutina nigrithorax*

In table 1 are reported the DWV copies number detected by RT-qPCR in *V. velutina nigrithorax* expressed as DWV copies/ng of input RNA.

No DWV was detected in the *V. velutina nigrithorax* foragers sampled in Airole area in early-season. The RT-qPCR has allowed the detection of DWV RNA from all samples collected in mid- (July in Bordighera, Sanremo and Dolceacqua) and in late-season (November in Giardini Hanbury, Ventimiglia). The viral load ranged from less than 10 (pool from Sanremo area) to 4.61×10^6 copies/ng RNA (in gynes collected in late-season).

Detection of replicating DWV in *V. velutina nigrithorax*

In samples collected in Sanremo and Bordighera in mid-season, although the virus was present, no replicative form was found. Instead, in samples collected in Dolceacqua, negative and positive ssRNA have been found demonstrating active viral replication.

In one female and two males collected in Ventimiglia in late-season, although the virus was present, no replicative form was found. Instead, in two gynes and one male have been found active viral replication by strand-specific RT-PCR.

Sequence analysis

Sequences obtained from strand specific RT-PCR positive samples indicated high similarity (99%) to DWV genome sequences available in GenBank. Pairwise distance analysis indicates the highest homology to DWV type A (table 2).

Discussion

Recent invasion of *V. velutina* in European countries has raised concern among beekeepers. In view of a global control strategy, the achievement of a new-

Table 1. DWV copies detected by RT-qPCR and positive/negative strand specific RT-PCR in pools of *V. velutina nigrithorax* samples collected in early- and mid-seasons and *V. velutina nigrithorax* specimens collected in late-season.

Sampling Season	Sample	DWV copies/ng RNA	Strand specific RT-PCR
Early-Season	Pool 1	negative	−
	Pool 2	negative	−
	Pool 3	negative	−
Mid-Season	Pool Dolceacqua	2.35×10^2	++
	Pool Bordighera	6.80×10^2	+
	Pool Sanremo	less than 10	+
Late-Season	Gyne 1	1.80×10^3	++
	Gyne 2	1.02×10^3	+
	Gyne 3	4.60×10^6	++
	Male 1	9.40×10^2	+
	Male 2	8.96×10^3	+
	Male 3	6.67×10^3	++

(−), negative sample; (+), detection of positive strand of DWV; (++), detection of replicative strand of DWV.

Table 2. Estimates of evolutionary divergence between DWV sequences.

	DWV type A NC_004830	DWV type B NC_006494	DWV type C CEND01000001
DWV type B NC_006494	14.165		
DWV type C CEND01000001	16.825	23.091	
V. velutina MH223315 - MH223316	0.012	14.245	16.920

The number of base substitutions per site from between sequences are shown. Analysis was conducted using the Maximum Composite Likelihood model on a total of 91 positions in the final dataset.

equilibrium between pathogen and the Asian hornet assumes great significance. A potential indicator of a new-equilibrium between viral pathogens and *V. velutina* could be represented by replicative virus/non-replicative virus ratio.

In *V. velutina*, the presence of a new *Iflavirus* namely Moku virus was detected (Garigliany *et al*., 2017; Mordecai *et al*., 2016a) and the infection by replicative Israeli acute paralysis virus (IAPV) was found (Yañez *et al*., 2012).

The wet weight of the late-season (November) newly-emerged females indicate that these specimens could be gynes, according to the wet weight reported by Rome and colleagues from future founder queens collected in November (Rome *et al*., 2015).

In this investigation, DWV RNA has been detected in *V. velutina nigrithorax* samples during their predatory activity in mid-season (July) and in those newly-emerged reproductive hornets sampled from their nest in Giardini Hanbury, in late-season (November). One pool, out of three, of free ranging hornets and three specimens, out of six, of newly-emerged hornets has shown active replication (presence of negative strand RNA) of DWV in both seasons of sampling. Replication of DWV in both *V. velutina nigrithorax* free ranging and of newly-emerged ones clearly demonstrate that DWV can infect also the exotic hornet thereby confirming what had been speculated by some of the authors of the present investigation in a previous paper concerning *V. crabro* DWV infection (Forzan *et al*., 2017b).

DWV has been detected in several bumblebees species (Tehel *et al*., 2016; Gisder and Genersch, 2017), in *Ceratina* spp. (Singh *et al*., 2010), in *Andrena* spp. (Singh *et al*., 2010), in *Augochlora pura* (Say) (Singh *et al*., 2010), in *Xylocopa virginica* L. (Singh *et al*., 2010), in *Osmia bicornis* (L.) (Ravoet *et al*., 2014), in *Bembyx* sp. (Singh *et al*., 2010), in several wasps of genus *Polistes* (Singh *et al*., 2010), in *Vespula vulgaris* (L.) (Evison *et al*., 2012; Singh *et al*., 2010) and *Vespula germanica* (F.) (Evison *et al*., 2012). DWV replicative form and evident symptoms were described only in several bumblebees species (Tehel *et al*., 2016; Gisder and Genersch, 2017) and *Osmia cornuta* (Latreille) (Mazzei *et al*., 2014; Ravoet *et al*., 2014).

Excluding Apoidea superfamily, the detection of asymptomatic DWV infection in the Asian hornet is in accordance with results obtained for the European hornet *V. crabro* (Forzan *et al*., 2017b) and for the Argentine ant *Linepithema humile* (Mayr) (Sébastien *et al*., 2015). DWV replicative form was reported only in 1 out of 15 *L. humile* (~ 7%) (Sébastien *et al*., 2015). This value is the same of the 1 out of 3 pools (if only 1 out of 15 specimens is considered positive) reported in this investigation in mid-season and is much lower than 3 out of 6 specimens (50%) reported in late-season *V. velutina nigrithorax* samples.

Despite the percentage differences of DWV replicative form between Asian hornet and Argentine ant, the capability of DWV to infect these two "new" hosts in its replicative form is demonstrated. The replicative infection differences could be due to the potential DWV titer, low in bee products raided by the ant from beehive (Sébastien *et al.*, 2015) and higher in honey bee specimens directly predated by the Asian hornets.

The detection of both non-replicative and replicative DWV among *V. velutina nigrithorax* hornets sampled in different areas, time and physiological state could be explained by the adaptation necessities of RNA viruses towards a new host (Ferrer-Orta *et al*., 2015; Morley *et al.*, 2015) as suggested for DWV by Forzan *et al*. (2017b).

DWV genome detected in *V. velutina nigrithorax* samples proved to belong to the worldwide diffused and less virulent genetic variant, DWV type A, as also reported for *V. crabro* (Forzan *et al*., 2017a).

In this investigation, the infection rate of Asian hornet by DWV ranges from 0 (early-season), to 33% (mid-season) and 50% (late-season). The viral load identified in *V. velutina nigrithorax* is comparable to values measured in asymptomatic honey bees (from 10^2 to 10^4 copies/ng RNA) and lower than the value measured in symptomatic honey bee (> 10^7 copies/ng RNA) (Mazzei *et al*., 2014). Although the seasonal increasing of hornet DWV infection rate was not the focus of this investigation and the number of samples was very few, results obtained in this investigation suggest that honey bee DWV infection could vary throughout the seasons, showing an increase in level of infection from spring to autumn (Porrini *et al*., 2016). Variation of DWV titre detected in *V. velutina nigrithorax* throughout the season is compatible with those detected in the honey bee colonies. Therefore, in late summer/early autumn there is a higher infection probability of *V. velutina nigrithorax* larvae by feeding them with DWV infected honey bee thoraxes. Moreover, both results i) detection of Asian hornet male infection by DWV and ii) the high

infection rate in late summer/early autumn, suggest that DWV is able to infect the reproductive hornets, allowing to hypothesize also a vertical transmission. These results open a new ecological perspective due to a potential active role of DWV in a new equilibrium prey (honey bees)/predator (Asian hornets) relationship.

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