

Varroa destructor and viruses association in honey bee colonies under different climatic conditions

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Summary

Honey bee colonies are threatened by multiple factors including complex interactions between environmental and diseases such as parasitic mites and viruses. We compared the presence of honeybee-pathogenic viruses and *Varroa* infestation rate in four apiaries: commercial colonies that received treatment against *Varroa* and non-treated colonies that did not received any treatment for the last 4 years located in temperate and subtropical climate. In addition, we evaluated the effect of climate and *Varroa* treatment on deformed wing virus (DWV) amounts. In both climates, DWV was the most prevalent virus, being the only present virus in subtropical colonies. Moreover, colonies from subtropical climate also showed

reduced DWV amounts and lower *Varroa* infestation rates than colonies from temperate climate. Nevertheless, non-treated colonies in both climate conditions are able to survive several years. Environment appears as a key factor interacting with local bee populations and influencing colony survival beyond *Varroa* and virus presence.

Introduction

Apis mellifera plays an important role in global economy as honey producer and as the main pollinator of food crops (Decourtye *et al.*, 2010). Honey bee colonies are threatened by a wide variety of diseases and parasites, including bee pathogenic viruses (Brutscher *et al.*, 2015). Growing attention is being paid to viruses infection since the increased number of colony losses seems to be explained by a combination of variables including co-infections with *Varroa destructor* (Genersch and Aubert, 2010; Francis *et al.*, 2013). Viruses differ in their geographical distribution (De Miranda *et al.*, 2010; Genersch and Aubert, 2010). Primarily, deformed wing virus (DWV) is globally distributed (De Miranda and Genersch, 2010) and its presence was linked to colonies losses (Francis *et al.*, 2013). Acute bee paralysis virus (ABPV) was linked to colony losses in Europe while the Israeli acute paralysis virus (IAPV), and Kashmir Bee Virus (KBV) were proposed as the causes of colony losses in United States (Genersch and Aubert, 2010). Together with these viruses, chronic bee paralysis virus (CBPV), black queen cell virus (BQCV) and sacbrood virus (SBV) were also detected in Argentina (Reynaldi *et al.*, 2010; 2011; Castilla *et al.*, 2015). On the contrary, the Kashmir bee virus (KBV) has been detected mostly in North America and New Zealand (De Miranda *et al.*, 2010) but so far no detection has been reported in Argentina or other countries from South America (Antúnez *et al.*, 2006; Teixeira *et al.*, 2008; Reynaldi *et al.*, 2010; 2011).

Varroa destructor plays an important role in the transmission and virulence of DWV. The mite causes a suppression of the immunocompetence of the host, giving to this virus the opportunity to infect bees (Yang and Cox-Foster, 2005). Besides, chemical treatment not only directly affects the immunity of the honey bees (Boncristiani *et al.*, 2012; Locke *et al.*, 2012) but also has an

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important influence on parasite–host relationship. Active Varroa control by beekeepers would disrupt any association between Varroa infestation rates and the virus epidemics (Mondet *et al.*, 2014).

Abrupt losses may occur triggered by the combination of these diseases with environmental factors such as climate conditions. Colony life histories including Varroa management, related to brood-free periods during periods without flowering, have a significant influence on Varroa infestation rates and consequently can affect associated virus presence or distribution (Meixner *et al.*, 2015).

The objective of this study was to evaluate the effect of climate and Varroa mites treatment on the prevalence of the main viruses present in honey bee colonies from Argentina.

Materials and methods

The presence of seven virus species (DWV, ABPV, CBPV, BQCV, KBV, IAPV and SBV) was evaluated in 25 colonies distributed in two different climatic conditions from Argentina: 13 in the subtropical climate and 12 in the temperate climate. In each climate, there were also compared Varroa infestation and viruses prevalence between commercial colonies (six per climate) that received annual treatment against Varroa mites ('Treated colonies' from now on) and 'non-treated' colonies (six in temperate and seven in subtropical) that did not receive any treatment against Varroa mites. These non-treated colonies were set up in 2011 from brood nuclei and selected queens from a network of evaluation of honeybee stocks for Varroa tolerance (Merke *et al.*, 2014a). This network is funded by the Beekeeping Program from the National Institute for Agricultural Technology (PROAPI-INTA) and it was originated from the genetic improvement program for honey bee brood diseases (Palacio *et al.*, 2000; 2003; 2012). Since 2007, this program had been also selecting colonies with Varroa tolerant traits following the same breeding protocol. Queen selection and stock reproduction was based on the capacity of the colonies to reduce Varroa population growth, either by defensive behaviour or mite reproduction interruption under different climate conditions (Merke *et al.*, 2014b). All samples were taken from asymptomatic colonies just before beekeepers applied the Varroa treatments to the treated colonies group at the beginning of autumn 2015 (late March).

The subtropical climate colonies were located in a region with annual mean temperature of 19.9°C (max: 25.9°C and min: 14.5°C) and mean annual precipitations of 1408 mm (29°14'00''S 59°56'00''W). The most relevant production is intensive livestock (dairy farms and wintering animals on alfalfa-based pastures) combined

with sunflower, sugar cane and cotton crops and natural forest. Temperate climate colonies were located in a region with annual mean temperature of 18°C (max: 25°C and min: 12.1°C), with average annual precipitations under 800 mm (31°16'00''S 61°29'00''W). The most relevant production is also intensive livestock but it is combined with agriculture production based on extensive soya, wheat, and corn crops (Giorgi *et al.*, 2008).

Sampling and virus analysis

About 40 worker bees were collected alive from the central frames of the brood chamber in each colony. Samples were immediately freezer frozen (within 2 h) and sent to the laboratory where they were macerated in mortar and homogenized with 7 ml of pH 7 phosphate buffer (PBS). The mixture was centrifuged at 4500 rpm at 8°C for 45 min and the supernatant was collected and stored at –20°C.

Pools of 30 bees were homogenized in 15 ml PBS and total RNA was extracted using TRIzol® Reagent (Invitrogen) following the manufacturer recommendation. RNA samples were dissolved in 10–50 µl ultra-pure water (Distilled Water DNase, RNase Free; Invitrogen).

Real Time PCR (RT-qPCR) was carried out to determine the presence of DWV, BQCV, ABPV, CBPV, IAPV and KBV. Copy DNA was synthesized by reverse transcription reaction from the extracted RNA. The reaction mixture contained 1 µl of RNA, 1 µl of reaction buffer 5x (Promega), 0.5 µl dNTP 10 mM (Promega), 0.125 µl of ARNsin 40U/µl (Promega), 0.25 µl of random primers 2 µg/µl, 0.175 µl of reverse transcriptase 200 U/µl (Promega) and completed with volume of 1.95 µl of ultra-pure water (Distilled Water DNase, RNase Free; Invitrogen) to obtain a total volume of 5 µl of mixture. The reaction was developed in a Biometra Trio-Thermoblock. The thermal cycling profiles were: 42°C for 45 min, 94°C for 10 min and 4°C for 4 min.

For the RT-PCR amplification, the reaction mixture contained 0.4 µl 1.5 µM of each pair of primers selected and described by Locke *et al.* (2012), 2.5 µl Master mix SYBER green PCR kit QuantiTect (cat 204143), 1.45 µl ultra-pure water (Distilled Water DNase, RNase Free; Invitrogen) and 0.5 µl of cDNA. Samples were amplified using the LightCycler 2.0 Roche Thermocycler with the following thermal cycling profiles: 95°C for 10 min, 45 cycles at 95° for 15 s and 56°C for 1 min. The fluorescence emission of the samples was performed at 530 nm. Samples having a geometric increase in fluorescence emission in the two previous successive cycles of cycling number 45 were considered positive. It was considered the first of these emission lifting cycles as first cycle of positivity. Negative (H₂O) and positive control (recombinant plasmid DNA with virus insert into

pGEM-T Easy vector) were included in each run of the RT-PCR reaction. Quantification of DWV was performed by RT-qPCR with the reference gene *DWVgp1* according to Chen *et al.* (2005). The estimation of the viral loads of positive samples was performed using standard curves prepared with threshold cycle (Ct) data obtained for known concentrations of cDNA fragments copies of the virus studied.

Sampling and Varroa analysis

Adult bees were examined to diagnose the presence of Varroa mites in all tested colonies. Approximately 250 bees per colony were collected from both sides of three unsealed brood combs in a jar containing 50% ethanol. The mites were separated from the bees by pouring the jar content into a sieve with a 2 mm mesh size (Dietemann *et al.*, 2013). The intensity of mite infestation on adult bees was calculated dividing the number of mites counted by the number of bees in the sample to determine the proportion of infested individuals and multiplying by 100 to obtain the infestation rate per colony (Dietemann *et al.*, 2013). In addition, the number of adult bees and number of cells with pollen and honey reserves of all colonies were estimated according to the Liebfeld method (Imdorf and Gerig, 2001).

Statistical analysis

Only DWV amounts were statistically assessed as it was the most prevalent virus and was found in all groups. The mite infestation rate and DWV amounts between subtropical and temperate climate and between treated and non-treated colonies were compared using a T student-test. DWV amounts was analysed with full factorial ANCOVA using climate (subtropical/temperate) and Varroa treatment (yes/no) as fixed factors and mite infestation rate as covariate. Since is not possible to log transform zero values, the response variable was Log_{10} of (DWV copies + 1) in order to include all values (negative and positive samples).

Results and discussion

In both climate DWV was the most prevalent virus being present in 3 of 13 and 12 of 12 colonies in subtropical and temperate climate, respectively ($P < 0.0001$). Colonies from subtropical climate showed only infections with DWV. On the contrary, in temperate climate 3 of 12 of the colonies had co-infection with BQCV and 3 of 12 with ABPV. Similarly, 1 of 12 colonies had been co-infected with CBPV and 1 of 12 with SBV also in colonies from temperate climate. No colonies tested positive for KBV or IAPV. Both treated and untreated colonies from temperate climate had co-infections. Colonies from temperate climate presented higher infestation rate with

Varroa mites ($9.32\% \pm 8.55\%$) than subtropical colonies ($2.49\% \pm 2.54\%$) ($t = 2.75$; $P = 0.01$). Varroa infestation rate was higher in treated than in non-treated colonies from subtropical climate ($t = -2.97$; $P = 0.01$) while it was similar in both groups from temperate climate ($t = -0.67$; $P = 0.52$). Mean mite infestation rate in colonies without virus was $2.04\% \pm 1.99\%$, while colonies with single DWV infection had $7.39\% \pm 4.29\%$ and colonies with co-infected DWV had $9.02\% \pm 10.59\%$ ($F = 2.85$; $P = 0.07$). No differences were found in mite infestation rate between colonies with and without the other detected viruses. Also, adult bee population and honey reserves were similar in all groups ($F = 1.059$; $P = 0.39$ and $F = 0.36$; $P = 0.783$, respectively). On the other hand, pollen cells in treated and non-treated colonies from temperate climate were significantly lower than pollen cells from the subtropical climate colonies ($F = 6.9$; $P = 0.002$).

The presence of BQCV, DWV CBPV, SBV and ABPV has been previously reported in Uruguay (Antúnez *et al.*, 2005; 2006); Brazil (Teixeira *et al.*, 2008) and Argentina (Reynaldi *et al.*, 2010). With the exception of DWV, viruses prevalence reported here were similar to preceding reports in Argentina (Castilla *et al.*, 2015) and lower than Viruses prevalence in France (Tentcheva *et al.*, 2004), Denmark (Francis *et al.*, 2013) and Uruguay (Antúnez *et al.*, 2006). Previous studies also reported the presence of IAPV in Argentina (Reynaldi *et al.*, 2011; Castilla *et al.*, 2015) although samples from both climates in this study were not infected with this virus or with KBV.

High prevalence and more species diversity were found in both groups of colonies from temperate climate suggesting an influence on infection prevalence (Meixner *et al.*, 2014). As temperate colonies showed also higher infestation rate with Varroa mites than subtropical colonies, it seems possible that it favours the occurrence of more than one virus species simultaneously. Mondet *et al.* (2014) suggested that the presence of Varroa increases the number of viruses that can be detected in a colony. Co-infection of DWV with other species occurred when varroa infestation was over 9%. Varroa mites are associated to ABPV and DWV occurrence (Ball and Allen, 1988; Bowen-Walker *et al.*, 1999; Chen and Siede, 2007) but transmission of BQCV and CBPV by varroa mites appears to be less probable (Tentcheva *et al.*, 2004; Chen and Siede, 2007). Nevertheless, viruses whose active transmission by Varroa is less certain still may benefit from Varroa weakened colonies (Mondet *et al.*, 2014; Amiri *et al.*, 2015).

Colonies from temperate climate showed higher DWV amounts compared with subtropical climate ($t = 6.86$; $P < 0.0001$); (Fig. 1). Similar DWV amounts was found between treated and non-treated colonies from

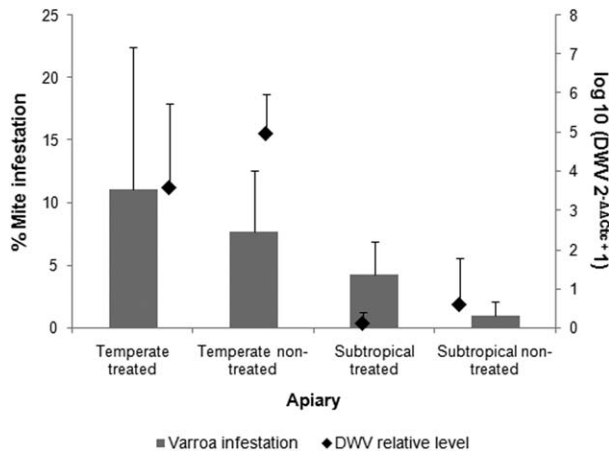


Fig. 1. Deformed wing virus relative virus level and mite infestation rate in honey bee colonies treated and non-treated against *Varroa destructor* from temperate and subtropical climate.

Table 1. Full factorial ANCOVA for Deformed Wing Virus (DWV) amounts in temperate and subtropical climate for honey bee colonies with and without annual treatment against *Varroa destructor*.

	F	df	P-value
Model intercept	25.62	1	< 0.001
Climate	32.74	1	< 0.001
Varroa treatment	4.57	1	0.045
Climate x Varroa treatment	0.76	1	0.39
Varroa infestation rate (covariate)	2.27	1	0.15

Levene's test F: 2.71; P = 0.07.

temperate climate ($t = 1.41$; $P = 0.19$) and from subtropical climate ($t = 0.96$; $P = 0.36$). DWV amounts in the autumn of 2015 were significantly influenced by climate and secondarily by the treatment against *Varroa* mites (Table 1).

Deformed Wing Virus appears to be the most prevalent virus in honey bee colonies from Argentina independently of the climate. As in previous studies, DWV was more common than other viruses such as ABPV (Tentcheva *et al.*, 2004; Meixner *et al.*, 2014). However, there were noticeable differences in DWV prevalence and amounts as well as in *Varroa* infestation rates in temperate and subtropical colonies (Lodesani *et al.*, 2014; Meixner *et al.*, 2014). One possible explanation may be related to a better nutritional source provided by subtropical climate as the pollen reserves in these colonies were significantly higher than in temperate colonies. As previously reported, nutritional status has an outstanding impact on colony health (Alaux *et al.*, 2010; Giacobino *et al.*, 2014; DeGrandi-Hoffman *et al.*, 2016). When climate is included in the analysis, we found a less significant contribution of the autumn infestation level of *Varroa* mites to DWV amounts (Meixner *et al.*, 2014). Environmental factors, particularly climate and

landscape may play a key role in mediating the host-parasite interaction, and perhaps honey bee health in general (Muli *et al.*, 2014).

DWV is known to be associated with *Varroa destructor* and has been detected in the mites (Genersch and Aubert, 2010). Heavy infestation during winter of either *Varroa* mites or DWV spread by the mite has been shown to be highly predictive of colony failure (Dainat *et al.*, 2012). The lower infestation registered in subtropical climate may explain partially the differences in DWV amounts between geographical zones. At the same time, these differences in *Varroa* infestation may be supported by a higher impact of the Africanized bees in subtropical colonies (Sheppard *et al.*, 1991; Rosenkranz, 1999) as honey bee race play a crucial role in resistance to *Varroa* (Camazine, 1986). Recently, Straus *et al.* (2015) showed that colonies of *A. mellifera scutellata* that did not present any signs of disease or collapse and were developing normally in the presence of *Varroa* mites. Similarly, new parasites and pathogens invading honey bee populations in East Africa seem not to directly impacted on Kenyan bee populations (Muli *et al.*, 2014). More accurately, the lower *Varroa* levels in colonies from subtropical zone could be explained by the fact that Africanized bees in South America have higher levels of hygienic behaviour, higher levels of grooming mites off of adult bees and lower levels of mite reproduction on pupae than European bees (Camazine, 1986; Guzman-Novoa *et al.*, 1999). However, although they were not part of this study, previous results obtained in these apiaries showed that the proportion of Africanized bees in both places was similar (J. Merke, pers. comm.).

Climate type had a highly significant influence on the mite infestations and apparently may be more important than race (Moretto *et al.*, 1991). Climate effect on the number of *Varroa* mites might be explained by longer brood presence, however regardless of the temperate climate in most regions from Argentina there is no broodless period (Marcangeli *et al.*, 1992; Giacobino *et al.*, 2015). Yet, assuming that bees from subtropical climate had, in fact, a relative longer season with brood and consequently higher mite populations than colonies in temperate climate (Vetharanim, 2012), this does not explain why treated and non-treated colonies from subtropical climate presented similar DWV relative virus levels.

The non-treated colonies, for which yearly treatment is not required, were selected for their capacity to limited *Varroa* population growth and therefore it was expected to have lower *Varroa* infestation rate than treated colonies (Francis *et al.*, 2013; Merke *et al.*, 2014b). Moreover, treated and non-treated colonies from subtropical climate differed significantly in their *Varroa* infestation rate but DWV relative virus levels were similar in both

groups. On the contrary, Varroa infestation rate and DWV amounts were similar between both groups in temperate climate. It seems that environmental condition might influence colony-specific epidemic factors, so they can exhibit low levels of DWV even with significant Varroa infestation rates, and vice versa (Mondet *et al.*, 2014).

Non-treated colonies from temperate climate showed the highest Varroa infestation rate and DWV relative virus levels but however had survived for the last 4 years. Climatic and other environmental conditions have been demonstrated to influence on the mite infestation level a colony is able to tolerate (Meixner *et al.*, 2014) and therefore to affect the probability of colony survival under multiples stress factor such as co-occurrence with virus species. For instance, it was mentioned before the relationship between climate conditions, bee race and Varroa mite levels (Camazine 1986; Moretto *et al.*, 1991).

Colonies from subtropical climate showed reduced virus prevalence and DWV amounts together with lower Varroa infestation rates compared to colonies from temperate climate, independently of Varroa control management. However, non-treated colonies are able to survive several years under different stress level in both climate conditions, probably because local populations of bees show better survival in the presence of pathogens than introduced bees (Meixner *et al.*, 2015). Environment appears as a key factor interacting with local bee populations and influencing colony survival beyond Varroa and Virus presence.

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