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RESEARCH ARTICLE

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Does pollen diversity influence honey bee colony health?

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Abstract

Aim of study: Colony losses of the western honey bee *Apis mellifera* have increased alarmingly in recent years. These losses have been attributed to nutritional deficiency, environmental conditions, viral infection and the global presence of the ectoparasite mite *Varroa destructor*. Ensuring pollen availability may improve colony health, so the present study aimed to examine whether the diversity of pollen collected by the colony as well as landscape characteristics of apiaries influence colony health.

Area of study: Tenerife Island (Canary Islands, Spain).

Material and methods: Colonies at eight apiaries were sampled in late summer to determine colony strength, presence of varroa and load of DWV. Pollen was collected during six months and analyzed. Landscape of each apiary was spatially analyzed.

Main results: Pollen diversity did not correlate significantly with colony strength or the load of DWV, but it positively correlated with varroa levels. In contrast, DWV load correlated with varroa infestation, and both variables negatively correlated with colony strength. Weak colonies were located in landscapes with areas less suitable for bee nutrition.

Research highlights: These results suggest that DWV and varroa infection as well as landscape characteristics influence colony survival, while pollen diversity on its own does not seem to have direct relationship. Our findings highlight the usefulness of DWV and varroa as predictors of colony losses, and they suggest the need to carefully assess honey bee apiary location in order to ensure adequate nutritional resources.

Additional keywords: viruses; DWV; varroa; colony losses; nutrition; landscape.

Abbreviations used: BQCV (Black queen cell virus), DWV (Deformed wing virus), GEC (genome equivalent copies), IAPV (Israeli acute paralysis virus), RT-qPCR (real-time reverse transcription-polymerase chain reactions), SBV (Sacbrood bee virus).

Authors' contributions: SBA, MVR, JAR and JMSV designed the study. JAR performed the sampling. AB carried out the palynological research. SBA carried out the laboratory work. SBA and MVR drafted the manuscript. All authors contributed to the critical review of the results and approved the final version of the manuscript.

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Introduction

Growth and survival of honey bee colonies are strongly related to the availability of food resources and the suitability of land uses, climate, temperature and other factors for ensuring adequate nutrition (Keller *et al*., 2005; Brodschneider & Crailsheim, 2010). Malnutrition, together with starvation, is considered one of the leading causes for declining bee populations (Stanley, 1974; Naug, 2009), since nutritional stress can weaken the immune system, whose maintenance is costly (Field *et al*., 2002). It may also contribute to the alarming losses of honey bee colonies around the world in recent years, which likely reflect a complex mixture

of factors including malnutrition, environmental conditions, intensive beekeeping (Jacques *et al*., 2017), infection with viruses such as deformed wing virus (DWV) (Barroso-Arévalo *et al*., 2019), and the global presence of the ectoparasite mite *Varroa destructor* (vanEngelsdorp *et al*., 2009).

Honey bees obtain nutrients from nectar, which is mainly carbohydrates, and pollen, which provides proteins, amino acids, lipids, starch, sterols, vitamins and minerals (Stanley, 1974; Roulston & Buchmann, 2000). Pollen provides protein, which are essential for development of hypopharyngeal glands in adult worker bees and for production of royal jelly to support normal growth, reproduction function and brood rearing (Haydak, 1970; Standifer, 1980). Pollen is also important for the physiological metabolism (Alaux *et al*., 2011), optimal development of the immune response (Alaux *et al.,* 2010) and resistance to pathogens (Schmidt *et al*., 1987; Field *et al.,* 2002) and pesticides (vanEngelsdorp *et al.,* 2009). Therefore, lack of pollen results in poor physiological conditions and increased susceptibility to external threats at individual level, which can lead to into colony losses (Keller *et al.,* 2005). Therefore, to study the relationship between colony pollen collection and health would allow better knowledge of the role of this important factor involved in the current loss of honey bee populations.

While most studies have focused on whether the quantity of pollen intake influences honey bee health and resistance to disease, if these outcomes are also influenced by the quality, diversity or richness of pollen is unclear. The composition or relative amounts of amino acids in pollen differ across floral species, raising the question of whether these differences play a crucial role in maintaining an optimal immune response of protective effects by pollen (Di Pasquale *et al.,* 2013). Previous studies have revealed that a diet of diverse (polyfloral) pollens may lead to greater immune competence than a monofloral diet (Alaux *et al*., 2010). This poses the question of whether insufficient pollen diversity can compromise the honey bee immune system (vanEngelsdorp *et al*., 2009) and thereby render colonies more susceptible to pathogens that have been associated with colony losses around the world (Cox-Foster *et al.,* 2007; Cornman *et al.,* 2012; Kang *et al*., 2015). Therefore, the present study aimed to examine the possible effects of pollen diversity on honey bee colony health.

In addition to pollen diversity, the dissemination of pathogens in the apiary can strongly influence colony health (Carreck *et al*., 2010; Dainat *et al.,* 2012; Gisder & Genersch, 2017). Among pathogens related to colony losses, honey bee viruses are widespread and most of them cause covert, persistent infections (Evans & Hung, 2000), making them difficult to detect and eradicate. Environmental factors, nutritional deficiency or parasite infestation can lead to a stress status in the colony that promotes viral replication and makes the colony less able to resist viral infection triggering the appearance of clinical symptoms (Dainat *et al*., 2012; Dainat & Neumann, 2013). Some viruses are particularly important due to their distribution and high prevalence, such as black queen cell virus (BQCV) and sacbrood bee virus (SBV), and/or their virulence, such as Israeli acute paralysis virus (IAPV) and deformed wing virus (DWV). Remarkably, the combined presence of DWV and the widespread *V. destructor* mite correlate with colony collapse and have been proposed as colony survival markers (Carreck *et al.,* 2010; Francis *et al*., 2013; Kielmanowicz *et al*., 2015; Nazzi & Pennacchio, 2018).

Food quantity and diet composition have been shown to influence honey bee physiological and immunological functions (Di Pasquale *et al*., 2013). Consequently, the capacity to face infectious diseases and especially viral covert infections could potentially be affected by diet. Elucidating how pollen diversity influences the infection of bee viruses would help clarify the role of diet on colony health. Since nutritional deficiency can trigger this stress, we hypothesized that colonies with poor pollen diversity may be less healthy and, in the presence of DWV and/or varroa, more likely to suffer damage due to these pathogens. To test this hypothesis, we studied whether variations in pollen diversity in the diet influence the dynamics of four bee viruses and the appearance of viral symptoms in the colonies. Due to its importance as vector of viruses, dynamic of the *V. destructor* mite was also considered for this objective. A secondary objective was to identify geographical and climatic factors from apiary locations that are related to increase presence of viral symptoms, depopulation or high viral and/or varroa loads thus having a negative effect in colony health.

Material and methods

Study area

The island of Tenerife (Canary Islands, Spain) was selected to perform the study for three main reasons. First, it is a limited area that has high beekeeping activity (5% of Spanish hive census) (MAPA, 2018). Second, it offers very different environmental conditions for apiary location attending to differences in altitude and microclimates. Third, it has a diverse and characterised subtropical flora usable by honey bees (Henríquez Jiménez & Paricio Núñez, 1979). Specifically, nearly

100 of approximately 740 endemisms present in the island can be used by honey bees. The specific location of each apiary is shown in Fig. 1.

The study included eight apiaries. Five of them were located 500 m above sea level, while the other three were below that altitude. Different climatic areas were found at different altitudes: coastal zone was under 300 m; middle area was at 300-600 m; and alpine area was above 600 m. Apiaries C and H were located in the coastal zone, where precipitation is low and temperature high. Apiaries A, B and G were located in the alpine area, which registers the most rainfall on the entire island as well as low temperatures (Rodriguez-Franco & Cuevas, 2013).

Sampling design

We studied eight apiaries of *A. mellifera* (denominated A-H), all of which were maintained in natural environments. If there were crops, those were small family crops (Fig. 1), which are a common type of crop in Tenerife Island. They are small-size orchards of horticultural crops and fruit trees, in which the use of pesticides tend to be avoided. The apiaries had a size between 20 and 40 Langstroth hives, as is common in Tenerife´s beekeeping. At each apiary, three colonies were selected and sampled for honey bees and pollen, thus 24 colonies were included in the study. At each apiary, colonies selected were re-queened with queens of the same age and genetic characteristics, and standardized to have 6-7 frames covered with bees and 2-3 frames covered with capped brood, in order to select strong colonies. Pollen was collected during the spring and summer, from April to September of 2014, since rainfall, temperatures and flowering vary between these two seasons (Henríquez Jiménez & Paricio Núñez, 1979). Seven pollen collections were performed in apiaries A and E and nine collections in apiaries B, C, D, F, G and H. Trial scheme is shown in Fig. 2. Pollen pellets were collected from pollen traps that were closed 24 h before sampling. All pollen samples were collected at the same day of the month in all the studied colonies.

Colony strength was measured at the start (April 2014) and at the end of the study (September 2014) by the veterinary technician. At the end of the pollen study (September 2014), two types of honey bee samples were collected from each colony (adults and brood). A total of 48 samples (24 adult samples and 24 brood samples) were collected in sterile containers, were kept on dry ice for the transport and frozen at -80ºC until laboratory analyses. Adult samples consisted of approximately 50 adult bees that were carefully taken by hand from the hive entrance or the honey combs of each colony. Additionally, one portion of brood comb was taken from each colony. These samples were analysed to detect the presence of four bee viruses (DWV, BQCV, IAPV and SBV). Given that beekeepers selected strong colonies, we assumed that the initial state was homogeneous in all the apiaries and, therefore,

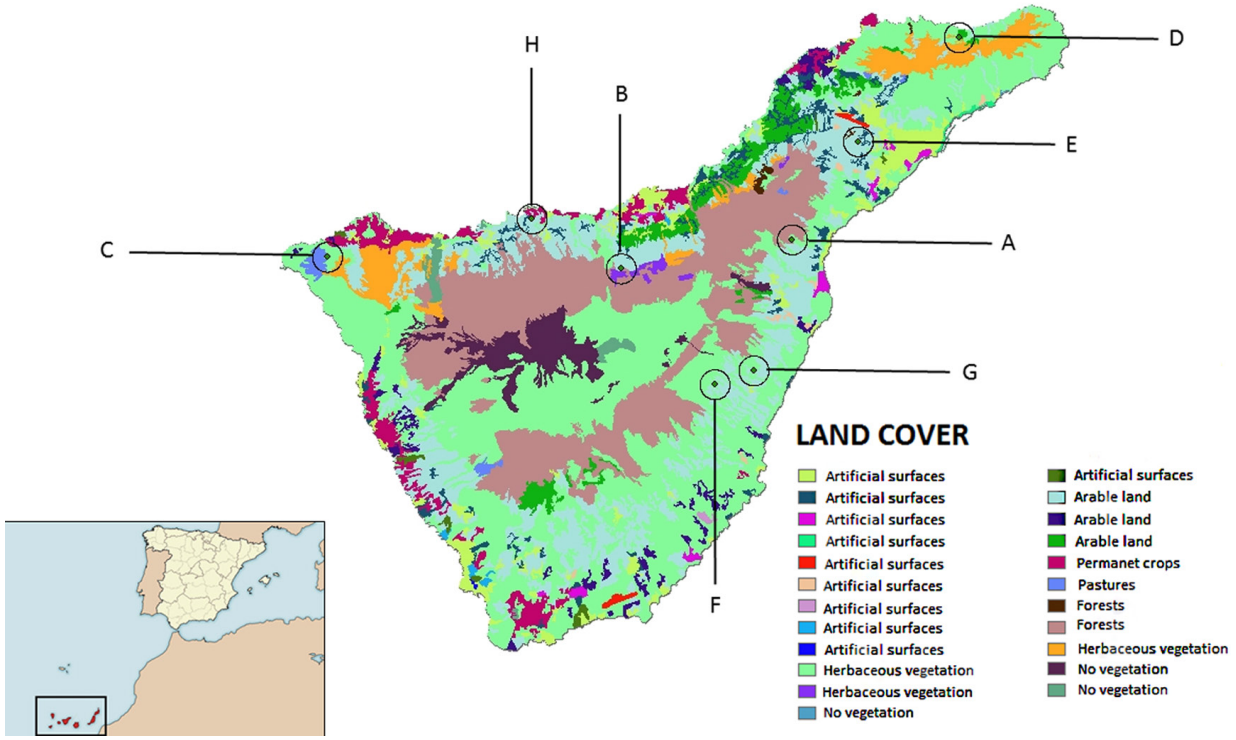

Figure 1. Location of apiaries in the study area.

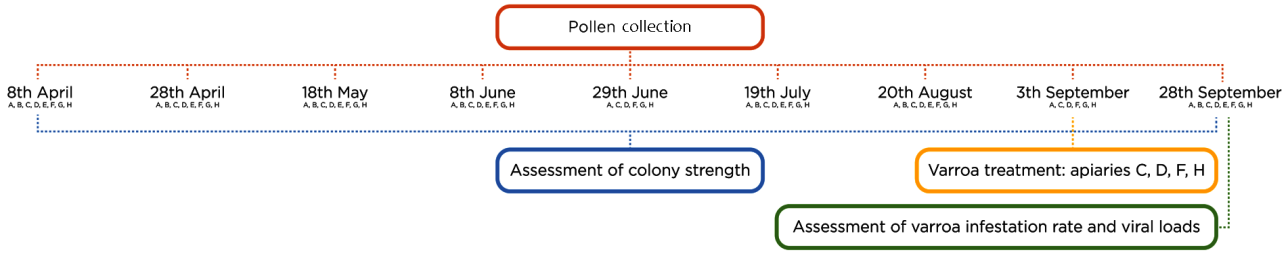

Figure 2. Schematic illustration of trial schedule and map. Three colonies in each site were requeened and equalized to have 6-7 bee frames and 2-3 brood frames. Colonies were sampled two times during the trial for hive strength, and one for bee viruses, and *Varroa destructor* counts. Pollen was collected nine times (apiaries A, C, D, F, G, H) and seven times (apiaries B and E).

we considered that at least one pathogen sampling could provide useful information about the evolution of the colonies in regard with pollen collection analysis. Regarding colony treatments, apiaries C, D, F and H were treated against *V. destructor* during the month before the honey bee sampling.

Assessment of bee population and symptoms

The honey bee population in each colony at the time of sampling was estimated by a veterinarian technician, who took into account the number of honey bee´s and brood´s combs (Delaplane *et al*., 2013). Colonies with signs of poor population (low activity in the entrance of the colony or fewer than five bee and brood combs were categorized as having a "poor population"; otherwise, colonies were categorized as having an "adequate population", considering the beekeeping managing and the time of the year. Health-related events were also observed in the sampled colonies, such as viral symptoms (deformity in wings and nervous symptoms), abnormal presence of *V. destructor* and mortality.

The presence of any of the health events already mentioned was considered together with the assessment of bee population in each colony. Colonies that showed at least one symptom and/or were classified as having a "poor population" were classified as "weak colonies", whereas colonies with an "adequate population" and without any symptom were classified as "healthy colonies".

Assessment of mite infestation levels

Mite infestation levels on adult honey bees were determined at bee sampling (on 28 September 2014). Mite infestation levels were calculated as the percentage of mites per 100 worker honey bees in the sampled colonies, using the soapy water method described in (Dietemann *et al.,* 2012). Briefly, 300 adult bees were collected from the colony from the sides of the unsealed brood combs, shaken in a tube containing soapy water and closed with a mesh top. In this procedure, mites detach from honey bee bodies and fall through the mesh. The percentage of mites was calculated as follows:

$$\% \text{ Infestation} = (\text{No. mites} / \text{No. bees counted}) \times 100\%$$

Detection and quantification of bee viruses

For each colony, separate pools of 10 honey bees and 10 pupae were crushed in a mortar in 5 mL of sterile phosphate-buffered saline (PBS) (Dietemann *et al*., 2013). This number of honey bees was used as we encountered difficulties in collecting pupae in some colonies. However, it allows detection of well-established viral infection in the colony as detection is performed with 95% probability of detection if at least 25% of honey bees are infected (Dietemann *et al*., 2013). This approach of detecting infections at an incidence of at least 25% is useful for *A. mellifera* studies because low viral load usually does not have a major impact on the colony for the viruses under study. In total, two homogenates were obtained from each colony. These homogenates were stored at -80 °C.

RNA was extracted from honey bees´s and pupae´s homogenates using the column-based Nucleospin II Virus® kit (Macherey Nagel) following the manufacturer's instructions. Total RNA was suspended in RNase- and DNase-free water and maintained at −20ºC until analysis. To ensure the correct preservation of RNA, RNA samples were analysed by one-step real-time reverse transcription-polymerase chain reactions (RT-qPCR) during the days following the extraction. After PCR analysis, samples were stored at -80 °C.

Samples were tested for presence and load of the following four bee viruses in separate one-step RT-qPCR based on SYBR-green detection as described: DWV and BQCV (Kukielka *et al.,* 2008a), IAPV (Maori *et al.,* 2009) and SBV (Amiri *et al*., 2015). Viral load in positive samples was quantified absolutely using a standard curve constructed with serial 10-fold dilutions of known amounts of plasmid DNA, starting from a cloned fragment of these virus (DWV and BQCV) into a PGemT® TA cloning vector (Promega) in accordance

with the manufacturer's instructions. The slope and intercept of each calibration curve were determined from a best-fit line with a correlation coefficient of 0.99. Viral loads were expressed in genome equivalent copies (GEC/µL) of homogenized bee sample and assigned to one of four categories of infection (Amiri *et al.,* 2015): virus-free (GEC=0); low virus titer, $0<GEC<10^3$; medium virus titer, $10^3 \leq GEC<10^7$; and high virus titer, $GEC \geq 10^7$. Plasmid confirmed as positive to each virus by PCR were used as a positive control for DWV, BQCV, SBV and IAPV.

Determination of pollen diversity

A pollen trap was placed at the hive entrance to collect pollen brought by foragers returning to the hive. Pollen was collected at 24 h after the trap had been closed, then sent by beekeepers to a central storage facility at the "Casa de la Miel" (Cabildo, Tenerife).

Pollen species and richness were established though palynological tests based on the method of Louveaux *et al*. (1978) with some variations. Briefly, pollen was diluted in distilled water, stained with glycerogelatin with basic fuchsine, and then examined under a microscope to determine species. Protein content was determined in quintuplicate using Kjeldahl analysis.

As a measure of diversity, we calculated the Shannon index, according to the following equation (Shannon & Weaver, 1949; Ramírez-Arriaga *et al.,* 2011):

$$H = -\sum_{i}^{n} pi \ln pi$$

where H = Shannon-Weaver diversity index; pi = proportion of each pollen type i encountered in the sample (counted in 500 grains of pollen per sample); and ln = natural logarithm.

Climatic and environmental parameters

Climatic and environmental conditions were studied for an area of radius 1,500 m around each apiary based on geographic coordinate system (GPS). This area is an effective foraging area of honey bees (Winfree *et al*., 2007). Land uses, forest cover, anthropogenic infrastructure, and landscape configuration and com-position were obtained from Corine Land Cover (Corine Land Cover, Version 17, 2006) and were represented geographically using GIS tools (ESRI, 2011). Temperature and precipitation data for Tenerife were obtained from the Weather Underground website (https://www.wunderground.com).

Habitat quality was measured in terms of six factors: number of land cover types (F1), number of important vegetable species for bees (F2), distance to permanent watercourses (F3), harvestable area unfragmented by infrastructures (F4), presence of animal farms near the study area (F5), and crop surface (F6). These factors were defined based on a literature review (Naug, 2009; Decourtye *et al.,* 2010; Gallant *et al*., 2014; Simioni *et al.,* 2015; Asensio *et al*., 2016) and the forestry map of the Canary Islands Government (Grafcan, 2015).

F1 was considered good when there were more than 5 land cover types. F2 was identified through an extensive literature research (Kunkel, 1991), and using the forestry map. Once the species were identified, protein composition of each species was used to classify them; the presence of more than 10 vegetable species for bees was considered the best condition for the study area. The absence of major roads, urban areas, and large bodies (coast) of water or industries around the study area was considered the best condition for F3. Closeness to rivers and streams (≤50 m) was considered the best condition for F4. The absence of animal farms near the study area was considered the best option for F5, as well as the absence of crop surface (F6).

Statistical analysis

Statistical analyses were performed using SPSS 22 (IBM, 2013). All data were stratified into 8 variables per colony and type of sample: 3 categorical (Apiary, A to H; Type, adult or brood; Health status, healthy or weak) and 5 continuous (DWV, BQCV, varroa, pollen richness and viral prevalence). Data for continuous variables were re-scaled into logarithmic scale.

Pathogen load was measured separately in adults and brood for each colony, and the higher load value was taken to be the value for the colony for the given pathogen. Pairwise comparisons in pathogen load between brood and adult samples were used, as well as between "weak" and "healthy", using the non-parametric Mann-Whitney U test (with a threshold of p-value <0.05). Potential relationships among pathogen loads and between pathogens and pollen richness were explored using Spearman's rank correlation.

A possible relationship between pollen richness and colony health category (weak, healthy) was explored using binomial logistic regression. Colony strength category was the dependent variable and was dichotomized as 1="weak colony" or 0="healthy colony".

Results

Viral load per colony and per sample

All samples tested negative for IAPV and SBV.

At the level of individual samples (adult and brood), DWV was present in 42 of 48 samples; load was high

in four samples, medium in 11, low in 24 and 5 samples were free of DWV. BQCV was detected in 35 of 48 samples; load was medium in 11 samples, low in 24 and 13 were free of virus. At the level of colony, DWV was detected in 22 of 24 colonies (91.66%), while BQCV was detected in 20 of 24 colonies (83.33%). SBV and IAPV were not detected. Average viral load was $<10^6$ GEC/µL (medium and low load) in 41 of 48 samples in the case of DWV, and in 45 of 48 samples in the case of BQCV.

At the level of apiary, only one of eight apiaries (apiary B) showed high DWV load, while no colonies showed high BQCV load. The load for both viruses was medium in the majority of cases (28 of 48 samples), especially in apiaries A, D and G, which were highly infested by varroa and had significantly higher DWV load (p=0.002) than the others. DWV load was lower in apiaries treated against varroa (p=0.001). DWV loads were significantly higher in brood samples with a varroa infestation level higher than 15% (p=0.005).

BQCV loads were significantly higher in adult samples than in brood samples (p=0.008). The results of DWV and BQCV load in adult and brood samples are summarized in Fig. 3. Therefore, colonies with highest BQCV load were likely to have fewer combs of brood (p=0.004).

Varroa destructor prevalence

Varroa was detected in 18 colonies (90%) at highly variable infestation levels from 0.35% to 42%; 11 of 18 positive colonies showed infestation levels <10%. Apiary B showed the highest infestation level, where mites were observed at this apiary during inspection, and the three colonies showed clear symptoms of infestation. Mite load correlated positively with DWV infection intensity (Spearman R=0.504, p=0.002). Apiaries treated against varroa showed significantly lower mite infestation levels than untreated apiaries (p<0.001).

Correlation between viral load, varroa infestation rate and pollen richness

In order to identify any potential relationships between viral load, varroa infestation rate and pollen richness and diversity, a spearman correlation analysis was performed. DWV load and varroa infestation rate showed significant correlation, as well as varroa infestation rate and pollen diversity, and pollen diversity and pollen richness. Results are shown in Table 1.

Honey bee populations and symptomatology

Six of the 24 colonies were classified as "weak colonies". These colonies belonged to apiaries B, D, E, F, G and H. The most frequently observed symptom was wing deformities, followed by visual detection of varroa in the colony. Based on binomial logistic regression, "weak colonies" showed significantly higher DWV load (odds ratio 1.665, p=0.015) and varroa infestation rate (odds ratio 1.059, p=0.044), compared to "healthy colonies".

Pollen richness and pollen diversity

Seventy-two plant species were identified in pollen samples, with the most frequent species indicated in Table 2 together with associated protein content.

Availability of pollen from diverse botanical origins (estimated through the Shannon index) varied along the period of study. Pollen richness varied throughout the period of study, ranging from 2 to 16; it was significantly higher in spring (April to June) than in

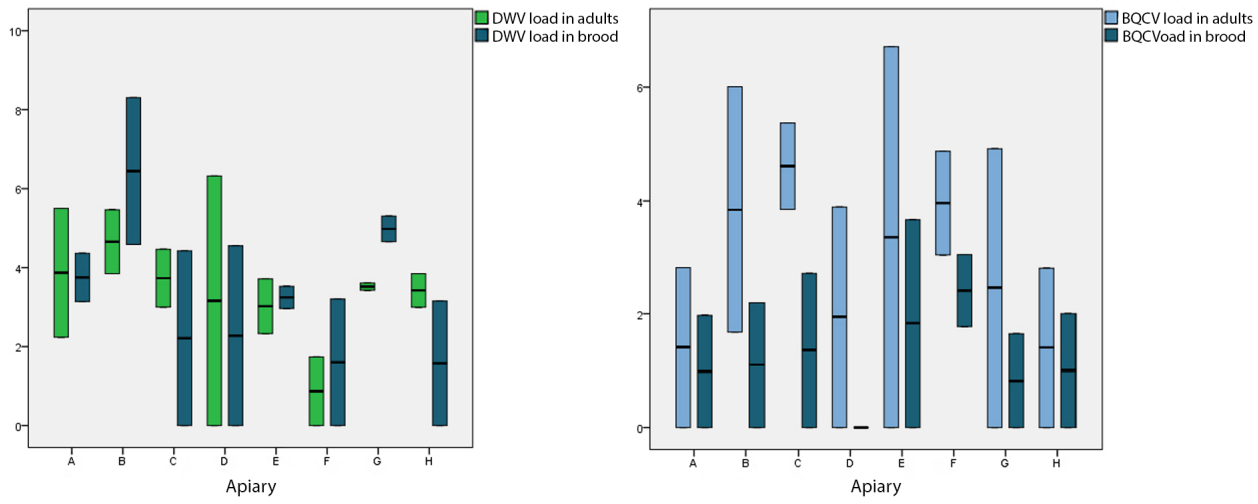

Figure 3. Log-transformed mean loads of DWV and BQCV (GEC/µL) in adult and brood samples at apiaries A to H.

Table 1. Spearman correlation analysis to identify relationships between pathogen load and pollen diversity.

Variables	Correlation coefficient	*p* value	N
DWV-BQCV	0.256	0.067	48
DWV-Varroa	**0.504**	**0.002**	**48**
BQCV-Varroa	-0.230	0.101	48
DWV-Pollen diversity	0.147	0.317	48
BQCV-Pollen diversity	0.189	0.594	48
Varroa-Pollen diversity	**0.384**	**0.012**	**48**
Pollen richness-Pollen diversity	0.771	>0.001	68

DWV: Deformed Wing Virus; BQCV: Black Queen Cell Virus. Significant results are highlighted in bold.

summer (June to September) (p=0.023). Shannon index ranged from 0.25 to 2.78 and no differences between seasons were detected (Fig. 4). Comparison of pollen richness and pollen diversity (Shannon index) in the last pollen collection (coinciding with pathogen assessment) is shown in Fig. 5.

Botanical species identified also varied along the study. In September, before honey bee sampling, the most frequent plant species were *Cistus monspeliensis, Kleinia neriifolia, Castanea sativa, Rubus ulmifolius* and *Galactites tomentosa*.

Environmental parameters

Varroa infestation was significantly higher in apiaries located 600 m above sea level (p<0.001).

Habitat conditions were worse (based on the six factors considered) around apiaries that were classified as "weak colonies" (*i.e*. dead honey bees around the colony, poor population) and that showed higher pathogen and varroa levels per apiary. These less favourable habitat conditions included the presence of urban areas, roads, abandoned fragmented bee habitat and reduced bee foraging area, all of which are associated with lower availability of natural resources. For example, more than one virus infected all colonies in apiary B, located near cattle pasture and the coast, factors that fragment the habitat; 2 of 3 colonies in this apiary had high DWV load, and one colony showed a varroa infestation level of 42%. In addition, the foraging area of this apiary contained two goat farms and one pig farm. Apiary D, which was located very close to the coast and had much of its foraging surface occupied by family crops, showed high DWV load and medium BQCV load, and it had weak colonies that showed symptoms (deformed wings) and poor population. In apiary E, dead bees around the colonies were observed, as well as other symptoms related to varroa and DWV presence (deformed wings). The foraging area contained crops, urban areas and cattle pasture, as well as 14 farms of pigs, cattle and goats (Grafcan, 2015). All these characteristics can fragment habitat, reducing the landscape heterogeneity and availability of food resources for honey bees.

Discussion

Malnutrition is one of the causes of honey bee losses, as it origins poor vigorous population and brood failure. Rarely is pollen entirely lacking from natural environments inhabited by honey bees, so the malnutrition that can drive colony losses is more a problem of poor pollen quality and diversity, and the latter has been related to nutritional quality (Alaux *et al*., 2010). Nutritional quality has been linked to adequate colony development (Brodschneider & Crailsheim, 2010). Therefore, we investigated whether pollen diversity accessible to honey bees at eight commercial apiaries on Tenerife Island (Spain) significantly affected colony health, which we measured in terms of appearance of symptoms, honey bee population, load of four honey bee viruses and varroa mite levels. Our results suggest that pollen diversity does not substantially influence colony health. Although varroa levels significantly correlated with pollen diversity, Spearman´s coefficient was weak (0.384). While these findings may reflect the fact that possible correlations between pollen diversity

Table 2. Most frequent plant species identified through pollen analysis, and the corresponding protein content.

Botanical species	Protein %
Aspalthium bituminosum	25.59
Brassica sp.	20.88
Carlina salicifolia	16.7
Castanea sativa	25.99
Chamaecytisus proliferum	25.72
Cistus symphytifolius	16.59
Citrus sinensis	14.74
Convolvulus floridus	18.87
Daphne gnidium	13.72
Echium plantagineum	35.34
Galactites tomentosa	19.75
Ilex canariensis	22.48
Lilium sp.	17.1
Papaver rhoeas	23.28
Papaver somniferum	23.95
Rubus ulmifolius	24.38
Rumex sp.	15.47

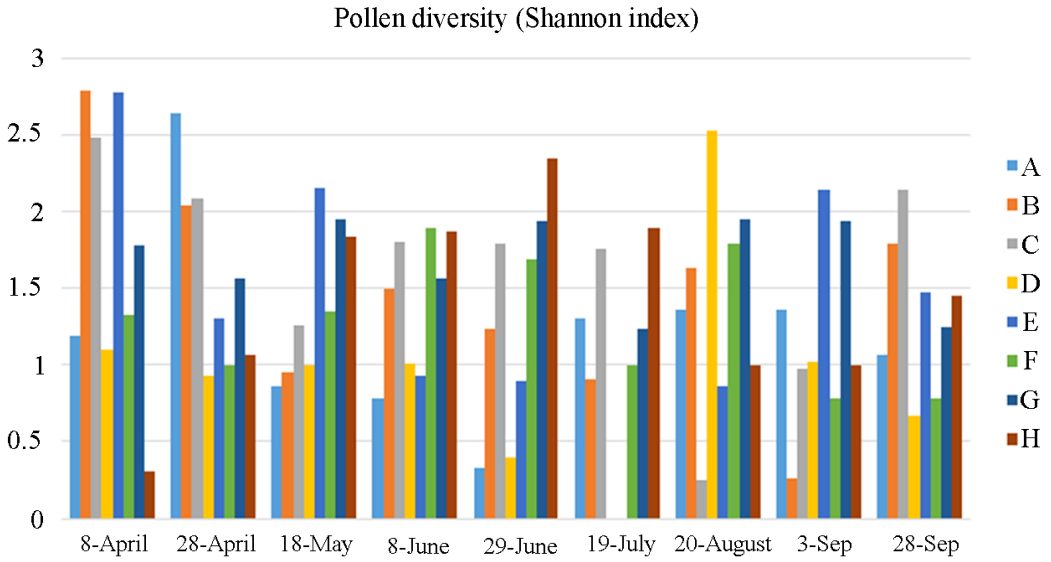

Figure 4. Seasonal variations of pollen diversity (Shannon index) per apiary.

and health parameters were tested only once (at the end of the summer), they may also indicate the need to focus on other factors that are more likely to play a strong role in determining honey bee health.

In our study, pollen diversity was higher in April; it fell at the beginning of the summer (end of June and July) and thereafter did not follow any clear pattern except that it remained lower than in the spring in almost all the apiaries. These results are consistent with the decline in bloom on the Canary Islands during the summer (Henríquez Jiménez & Paricio Núñez, 1979). Brood blocking and death of adult honey bees in this season contribute to lower foraging activity and

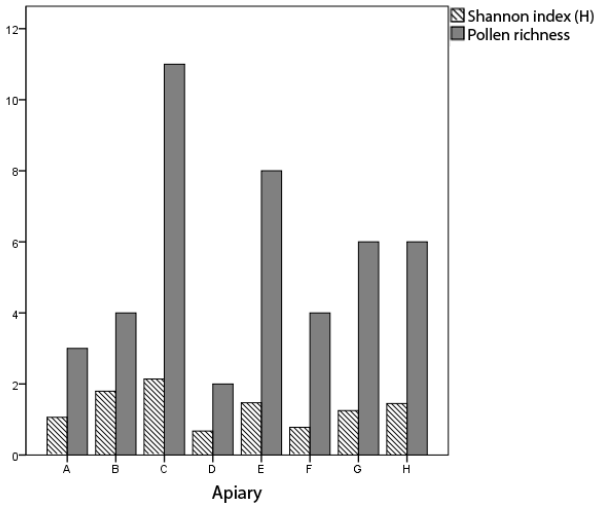

Figure 5. Comparison of pollen richness and diversity (Shanon index) in the last pollen collection (28 September), coinciding with the assessment of varroa infestation rate and viral loads.

therefore smaller colony population. Despite the higher pollen diversity in spring, and the association between higher diversity and protein content, we did not observe the positive correlation between pollen diversity and resistance to disease reported by Antunez *et al*. (2015). On the contrary, we found that higher pollen diversity correlated with higher varroa levels. A possible explanation for this might be that a more diverse diet may have a positive impact on individual and social immunocompetence and physiology, as suggested in previous works (Alaux *et al*., 2010, 2011; Di Pasquale *et al.,* 2013). As a consequence, it may be reflected in an increase of brood production, which would encourage mite reproduction (Evans & Cook, 2018). Further studies on this topic needs to be undertaken before the association between high pollen diversity and varroa levels is more clearly understood. Thus, these results suggest that at the apiaries in our study, and under the particular conditions of beekeeping in Tenerife, diverse diet by itself may not directly benefit colony health. Instead, other factors working alone or in conjunction with pollen diversity may influence colony resistance to disease.

Main plant species found in this study were *Cistus monspeliensis, Kleinia neriifolia, Castanea sativa, Rubus ulmifolius* and *Galactites tomentosa*, which are considered flora of bee interest (Silva & Restrepo, 2012). *C. monspeliensis* has high pollen content although relatively low protein content. *K. neriifolia,* which is endemic in Tenerife, is often used by honey bees, and it produces a large amount of pollen (Kunkel, 1991). *C. sativa* provides nectar and honeydew, its pollen has a high protein content (25.99%) and it is frequently the only food supply for honey bees. *R. ulmifolius* is a

source of nectar and pollen, especially when flowers bloom in summer, and it has high protein content. In contrast, *G. tomentosa* supplies nectar but relatively little pollen (Rodriguez-Franco & Cuevas, 2013). These findings show that Tenerife has a diversified vegetation for honey bee production.

Viruses can seriously affect colony survival (McMenamin *et al.,* 2018). They usually persist in colonies as covert infections, without causing obvious damage to honey bees. Under certain circumstances, viruses start replicating and infections can become overt and show symptomatology. Adverse environmental conditions (*i.e*., habitat, climatology), the varroa pressure and changes in the population structure of the colony might influence overwintering abilities of honey bees. In this scenario, opportunistic viral infections can promote and cause the decrease the survival ability of the colony. Therefore it was examined the load of four honey bee viruses in the apiaries of our study. Only two viruses were detected in the colonies: DWV was found in 91.66% of colonies, while BQCV was found in 83.33%. Most colonies had only low or medium load of these viruses, which is lower than the load measured at apiaries on the Spanish peninsula (Kukielka *et al.,* 2008b; Asensio *et al.,* 2016). Viral analysis was performed for adults and brood separately in order to detect potentially differential effects on the two populations. Only BQCV presented differences for the two populations, in which viral load was significantly higher in adults. This finding may be related to the viral ability to promote covert infections in the case of low pathogenic-viruses, being able to achieve high load in adults without causing mortality or any symptoms.

Both varroa infestation and DWV infection strongly influenced the health status of the apiaries in our study. Weak colonies, which showed symptoms or poor population, also had the highest levels of DWV and varroa. None of the colonies died during the study, and therefore no relationship between pathogens and colony collapse was found. However, this is consistent with previous studies that associated DWV load with colony weakness, suggesting that it may be a good predictor of colony weakness and losses, as it was reported by some studies (Cox-Foster *et al.*, 2007; Cornman *et al.,* 2012; Kang *et al.*, 2015; Kielmanowicz *et al.,* 2015). Correlation between DWV and varroa loads was found, echoing previous studies (Cornman *et al.,* 2012) and reflecting the fact that the mite effectively transmits viruses such as DWV (Ryabov *et al.,* 2014). By feeding on hemolymph, varroa can directly injure honey bees while at the same time transmitting viruses, activating viral replication and suppressing the immune system, rendering the honey bees more vulnerable to disease (Yang & Cox-Foster, 2005). This can give rise to synergism involving virus-parasite-host immunity (Di Prisco *et al.,* 2016), which can strongly alter the expression of several immune genes. These considerations suggest that DWV and varroa can act together to affect colony health, and that these negative effects are likely to be greater when brood is growing, since varroa is reproducing and thereby helping DWV to replicate. Further study is needed to clarify the roles of each pathogen in the development of colony weakness. Future work should also examine whether loads of these and other pathogens interact with pollen diversity, which negatively correlated with infection intensities of some viruses in previous work (Alaux *et al.,* 2011) but not in the present study.

Mite control could help avoid colony losses associated with varroa and varroa-transmitted viruses, as well as reduce risk of strong DWV infection in brood. Anti-varroa treatment of apiaries C, D, F, and H considerably reduced DWV load in colonies, indicating that this virus is not efficiently transmitted horizontally in the absence of mites (Kang *et al.,* 2015), and that vertical transmission routes are unlikely to lead to heavy loads in bee brood. Taking together, varroa infestation and DWV infection play a crucial role in the colonies. Monitoring viral load, especially DWV load, may help detect poor honey bee colony health and predict colony losses during overwintering.

In addition to pathogens, habitat conditions at the apiaries in our study strongly affected colony development: apiaries located near the coast showed worse population or symptomatology than other apiaries, and higher altitude was associated with lower colony strength. The diversity of habitat conditions across the apiaries provided a good opportunity for us to assess landscape effects. These findings emphasize the need for further research into effects of environment on honey bee colonies, and the need to take environment into account when lo-cating hives. Landscape features that reduce resource availability, such as proximity to pastures or urban areas and land fragmentation may increase risk of colony loss and risk of collapse during overwintering (Highfield *et al.,* 2009; Schroeder & Martin, 2012; Asensio *et al.,* 2016). Therefore, future studies should deepen on the understanding of environmental effect in honeybee colonies.

While the findings of this study should be treated with caution because of the limited sampling and pathogen analysis, these results nevertheless provide evidence that pollen diversity does not directly benefit honey bee colony health by itself. This may reflect the complex relationships among diet, breeding and pathogens: for example, colonies with access to abundant nutrients may breed rapidly, which at the same time allows rapid proliferation of varroa and the viruses that it transmits. In this way, changes in pollen diversity

may exert effects at the colony level without affecting health markers such as pathogen load. Another possible explanation for our observed lack of correlation between pollen diversity and colony health is that a key determinant is not so much the species diversity in available pollen, but the composition of specific amino acids in that pollen. Our analysis of overall protein content in the different pollens sampled suggests that protein supply did not vary substantially along the study period, but it is still possible that the availability of critical amino acids did vary, which in turn may have affected colony resistance to disease. A third possible explanation for the lack of correlation between pollen diversity and health markers is that a key determinant is the immune strength of the colony (Highfield *et al.,* 2009; Alaux *et al.,* 2010), which we did not analyze directly. Future work should examine whether changes in the intake of specific amino acids affect health markers, and whether this diet factor or even pollen diversity directly affect the immunocompetence of individuals and the colony as a whole.

As conclusion, this study suggests that pollen diversity in Tenerife Island does not significantly affect honey bee colony health or resistance to pathogens by itself, at least based on the analysis of data collected at the end of the summer. Instead, varroa parasitism and the presence of DWV exerted the greatest stress on the colony at this time. Therefore, these two factors should be taken into account for assessing colony health in future research.

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References

Agency EE, 2006. Corine Land Cover, seamless vector data (vers. 17). https://land.copernicus.eu/

Alaux C, Ducloz F, Crauser D, Le Conte, Y, 2010. Diet effects on honeybee immunocompetence. Biol Lett 6 (4): 562-565. https://doi.org/10.1098/rsbl.2009.0986

Alaux C, Dantec C, Parrinello H, Le Conte Y, 2011. Nutrigenomics in honey bees: digital gene expression analysis of pollen's nutritive effects on healthy and varroa-parasitized bees. BMC Genomics 12: 496. https://doi.org/10.1186/1471-2164-12-496

Amiri E, Meixner M, Nielsen SL, Kryger P, 2015. Four categories of viral infection describe the health status of honey bee colonies. PLoS One 10 (10): e0140272. https://doi.org/10.1371/journal.pone.0140272

Antunez K, Anido M, Branchiccela B, Harriet J, Campa J, Invernizzi C, Santos E, Higes M, Martín-Hernández R, Zunino P, 2015. Seasonal variation of honeybee pathogens and its association with pollen diversity in Uruguay. Microb Ecol 70 (2): 522-533. https://doi.org/10.1007/s00248-015-0594-7

Asensio I, Vicente-Rubiano M, Munoz MJ, Fernandez-Carrion E, Sanchez-Vizcaino JM, Carballo M, 2016. Importance of ecological factors and colony handling for optimizing health status of apiaries in mediterranean ecosystems. PLoS One 11 (10): e0164205. https://doi.org/10.1371/journal.pone.0164205

Barroso-Arévalo S, Fernández-Carrión E, Goyache J, Molero F, Puerta F, Sánchez-Vizcaíno JM, 2019. High load of Deformed Wing Virus and varroa destructor infestation are related to weakness of honey bee colonies in Southern Spain. Front Microbiol 10 (1331): 1-8. https://doi.org/10.3389/fmicb.2019.01331

Brodschneider R, Crailsheim K, 2010. Nutrition and health in honey bees. Apidologie 41 (3): 278-294. https://doi.org/10.1051/apido/2010012

Carreck NL, Ball BV, Martin S, 2010. Honey bee colony collapse and changes in viral prevalence associated with Varroa destructor. J Apic Res 49 (1): 93-94. https://doi.org/10.3896/IBRA.1.49.1.13

Cornman RS, Tarpy DR, Chen Y, Jeffreys L, Lopez D, Pettis JS, vanEngelsdorp D, Evans JD, 2012. Pathogen webs in collapsing honey bee colonies. PLoS One 7 (8): e43562. https://doi.org/10.1371/journal.pone.0043562

Cox-Foster DL, Conlan S, Holmes EC, Palacios G, Evans JD, Moran NA, Quan PL, Briese T, Hornig M, Geiser DM, *et al.,* 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318 (5848): 283-287. https://doi.org/10.1126/science.1146498

Dainat B, Evans JD, Chen Y, Gauthier L, Neumann P, 2012. Predictive markers of honey bee colony collapse. PLoS One 7 (2): e32151. https://doi.org/10.1371/journal.pone.0032151

Dainat B, Neumann P, 2013. Clinical signs of deformed wing virus infection are predictive markers for honey bee colony losses. J Invertebr Pathol 112: 278-280. https://doi.org/10.1016/j.jip.2012.12.009

Decourtye A, Mader E, Desneux N, 2010. Landscape enhancement of floral resources for honey bees in agro-ecosystems. Apidologie 41 (3): 264-277. https://doi.org/10.1051/apido/2010024

Delaplane KS, van der Steen J, Guzman-Novoa E, 2013. Standard methods for estimating strength parameters of *Apis mellifera* colonies. J Apic Res 52 (1): 1-12. https://doi.org/10.3896/IBRA/1.52.1.03

Di Pasquale G, Salignon M, Le Conte Y, Belzunces LP, Decourtye A, Kretzschmar A, Suchail S, Jean-Luc B,

Alaux C, 2013. Influence of pollen nutrition on honey bee health: do pollen quality and diversity Matter? PLoS One 8 (8): e72016. https://doi.org/10.1371/journal.pone.0072016

Di Prisco G, Annoscia D, Margiotta M, Ferrara R, Varricchio P, Zanni V, Caprio E, Nazzi F, Pennacchio F, 2016. A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health. P Natl Acad Sci USA 113 (12): 3203-3208. https://doi.org/10.1073/pnas.1523515113

Dietemann V, Ellis J, Neumann P, 2013. The Coloss Beebook, Vol I, standard methods for *Apis mellifera* research: introduction. J Apic Res 52: 1-4. https://doi.org/10.3896/IBRA.1.52.4.23

Dietemann V, Nazzi F, Martin S, Anderson D, Locke B, Delaplane K, Wauquiez, Tannahill C, Frey E, Ziegelmann B, Rosenkranz P, Ellis J, 2012. Standard methods for varroa research. J Apic Res 52 (1): 1-54. https://doi.org/10.3896/IBRA.1.52.1.09

ESRI, 2011. ArcGIS Desktop: Release 10. Environ Syst Res Inst, Redlands, CA, USA.

Evans JD, Hung AC, 2000. Molecular phylogenetics and the classification of honey bee viruses. Archiv Virol 145 (10): 2015-2026. https://doi.org/10.1007/s007050070037

Evans JD, Cook SC, 2018. Genetics and physiology of Varroa mites. Curr Opin Insect Sci 26: 130-135. https://doi.org/10.1016/j.cois.2018.02.005

Field C, Johnson I, Schley P, 2002. Nutrients and their role in host resistance to infection. J Leukoc Bio 71 (1): 16-32.

Francis R, Nielsen S, Kryger P, 2013. Varroa-virus interaction in collapsing honey bee colonies. PLoS One 8 (3): e57540. https://doi.org/10.1371/journal.pone.0057540

Gallant AL, Euliss NH, Browning Z, 2014. Mapping large-area landscape suitability for honey bees to assess the influence of land-use change on sustainability of national pollination services. PLoS One 9 (6): e99268. https://doi.org/10.1371/journal.pone.0099268

Gisder S, Genersch E, 2017. Viruses of commercialized insect pollinators. J Invertebr Pathol 147: 51-59. https://doi.org/10.1016/j.jip.2016.07.010

Grafcan, 2015. Geographical Information System of Canary Islands. http://visor.grafcan.es/visorweb

Haydak MH, 1970. Honey bee nutrition. Annu Rev Entomol 15 (1): 143-156. https://doi.org/10.1146/annurev.en.15.010170.001043

Henríquez Jiménez F, Paricio Núñez E, 1979. Manual práctico de apicultura para las Islas Canarias. Consejería de Agricultura y Pesca, Santa Cruz de Tenerife. https://mdc.ulpgc.es/cdm/ref/collection/MDC/id/43961

Highfield AC, El Nagar A, Mackinder LC, Noël LMLJ, Hall MJ, Martin S, Schroeder D, 2009. Deformed Wing Virus implicated in overwintering honeybee colony losses. Appl Environ Microbiol 75 (22): 7212-7220. https://doi.org/10.1128/AEM.02227-09

IBM, 2013. SPSS Statistics for Windows (vers 22.0). Armonk, NY. http://www-01.ibm.com/software/es/analytics/spss/

Jacques A, Laurent M, Consortium E, Ribière-Chabert M, Saussac M, Bougeard S, Budge GE, Hendrikx P, Chauzat MP, 2017. A pan-European epidemiological study reveals honey bee colony survival depends on beekeeper education and disease control. PLoS One 12 (3): e0172591. https://doi.org/10.1371/journal.pone.0172591

Kang Y, Blanco K, Davies T, Wang Y, 2015. Disease dynamics of honeybees with *Varroa destructor* as parasite and virus vector. Math Biosci 275: 71-92. https://doi.org/10.1016/j.mbs.2016.02.012

Keller I, Fluri P, Imdorf A, 2005. Pollen nutrition and colony development in honey bees-Part II. Bee World 86 (2): 27-34. https://doi.org/10.1080/0005772X.2005.11099650

Kielmanowicz MG, Inberg A, Lerner IM, Golani Y, Brown N, Turner CL, Hayes GJR, Ballam JM, 2015. Prospective large-scale field study generates predictive model identifying major contributors to colony losses. PLOS Pathogens 11 (4): e1004816. https://doi.org/10.1371/journal.ppat.1004816

Kukielka D, Esperón F, Higes M, Sánchez-Vizcaíno JM, 2008a. A sensitive one-step real-time RT-PCR method for detection of deformed wing virus and black queen cell virus in honeybee *Apis mellifera*. J Virol Meth 147 (2): 275-281. https://doi.org/10.1016/j.jviromet.2007.09.008

Kukielka D, Perez A, Higes M, del Carmen Bulboa M, Sánchez-Vizcaíno JM, 2008b. Analytical sensitivity and specificity of a RT-PCR for the diagnosis and characterization of the spatial distribution of three *Apis mellifera* viral diseases in Spain. Apidologie 39 (6): 607-617. https://doi.org/10.1051/apido:2008040

Kunkel, G, 1991. Flora y vegetación del archipiélago Canario. Tratado florístico, 2ª parte. Edirca, Las Palmas de Gran Canaria.

Louveaux J, Maurizio A, Vorwohl G, 1978. Methods of Melissopalynology. Bee World 59 (4): 139-157. https://doi.org/10.1080/0005772X.1978.11097714

Maori E, Paldi N, Shafir S, Kalev H, Tsur E, Glick E, Sela I, 2009. IAPV, a bee-affecting virus associated with Colony Collapse Disorder can be silenced by dsRNA ingestion. Insect Mol Biol 18 (1): 55-60. https://doi.org/10.1111/j.1365-2583.2009.00847.x

MAPA, 2018. El sector apícola en cifras. Principales indicadores económicos. Ministerio de Agricultura, Pesca y Alimentación, Spain.

McMenamin AJ, Daughenbaugh KF, Parekh F, Pizzorno MC, Flenniken ML, 2018. Honey bee and bumble bee antiviral defense. Viruses 10 (8): 395. https://doi.org/10.3390/v10080395

Naug D, 2009. Nutritional stress due to habitat loss may explain recent honeybee colony collapses. Biol Conserv 142 (10): 2369-2372. https://doi.org/10.1016/j.biocon.2009.04.007

Nazzi F, Pennacchio F, 2018. Honey bee antiviral immune barriers as affected by multiple stress factors: a novel paradigm to interpret colony health decline and collapse. Viruses 10 (4): 159. https://doi.org/10.3390/v10040159

Ramírez-Arriaga E, Navarro-Calvo LA, Díaz-Carbajal E, 2011. Botanical characterisation of Mexican honeys from a subtropical region (Oaxaca) based on pollen analysis. Grana 50 (1): 40-54. https://doi.org/10.1080/00173134.2010.537767

Rodriguez-Franco JJ, Cuevas E, 2013. Characteristics of the subtropical tropopause region based on long-term highly resolved sonde records over Tenerife. J Geophys Res: Atmospheres 118 (19): 10754-10769. https://doi.org/10.1002/jgrd.50839

Roulston TH, Buchmann S, 2000. A phylogenetic reconsideration of the pollen starch-pollination correlation. Evol Ecol Res 2: 627-643.

Ryabov EV, Wood GR, Fannon JM, Moore JD, Bull JC, Chandler D, Mead A, Burroughs N, Evans JD, 2014. A virulent strain of deformed wing virus (DWV) of honeybees (*Apis mellifera*) prevails after V*arroa destructor-mediated*, or in vitro, transmission. PLoS Pathog 10 (6): e1004230. https://doi.org/10.1371/journal.ppat.1004230

Schroeder D, Martin S, 2012. Deformed wing virus: The main suspect in unexplained honeybee deaths worldwide. Virulence 3 (7): 589-591. https://doi.org/10.4161/viru.22219

Schmidt J, C Thoenes S, Levin M, 1987. Survival of honey bees, *Apis mellifera* (Hymenoptera: Apidae), fed various pollen sources. Ann Entomol Soc Am 80 (2): 176-183. https://doi.org/10.1093/aesa/80.2.176

Shannon CE, Weaver W, 1949. The mathematical theory of communication. Univ. Illinois Press, Champaign, IL, USA.

Silva L, Restrepo S, 2012. Flora apícola: determinación de la oferta floral apícola como mecanismo para optimizar producción, diferenciar productos de la colmena y mejorar la competitividad. Instituto de Investigación de Recursos Biológicos Alexander von Humboldt, Colombia.

Simioni LC, Mussury RM, Mauad M, Dresh DM, Pereira FF, Scalon SPQ, 2015. Plant-pollinator interactions in *Crambe abyssinica* Hochst. (Brassicaceae) associated with environmental variables. Anais da Academia Brasileira de Ciências 87: 137-145. https://doi.org/10.1590/0001-3765201520130365

Standifer LN, 1980. Honey bee nutrition and supplemental feeding. Agriculture Handbook 335: 39-45. http://bulletswww.three-peaks.net/PDF/Honey%20Bee%20Nutrition%20and%20Supplemental%20Feeding.pdf

Stanley RG, 1974. Pollen: biology, biochemistry, management. Springer-Verlag, Berlin.

vanEngelsdorp D, Evans JD, Saegerman C, Mullin C, Haubruge E, Nguyen BK, Frazier M, Frazier J, Cox-Foster D, Chen Y, Unerwood R, Tarpy DR, Pettis JS, 2009. Colony collapse disorder: a descriptive study. PLoS One 4 (8): e6481. https://doi.org/10.1371/journal.pone.0006481

Winfree R, Williams NM, Dushoff J, Kremen C, 2007. Native bees provide insurance against ongoing honey bee losses. Ecol Lett 10 (11): 1105-1113. https://doi.org/10.1111/j.1461-0248.2007.01110.x

Yang X, Cox-Foster DL, 2005. Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. P Natl Acad Sci USA 102 (21): 7470-7475. https://doi.org/10.1073/pnas.0501860102