ABSTRACT

The honey bee *Apis mellifera* is the main pollinator species used to increase the productivity of crops and it also has a central role in the conservation of the worldwide diversity. In the last decades, an increase in the mortality of honey bee colonies has been observed in many regions, causing a great alarm due to its potential economic and environmental consequences. This phenomenon, which has unknown causes, has been called "Colony Collapse Disorder" (CCD) when a depopulation of adult honey bees is observed, or "winter losses" when colonies do not survive to the winter by unidentified causes. These losses have also been observed in Spain, country within the European Union with the highest hive census and with important honey productions. This situation has generated the need to discover the cause of these losses. Nowadays it is considered that no single cause can explain this mortality, but this is a phenomenon where the interaction of multiple factors can affect honey bee colonies. Among these 'risk' factors some are highlighted, such as poor nutrition and lack of food resources, adverse climatology, exposure to neonicotinoid pesticides used in crops where bees forage, presence of natural predators and invasive species and presence of pathogens in the colonies. Viruses are important pathogens not only for their extensive prevalence and distribution and their association with colony mortality, but for the unknown aspects about their pathogenicity, how viruses can be affected by other factors and how viruses can alter the balance with the host leading to a pathological status.

This doctoral thesis, entitled **"Virological and Epidemiological analysis of Colony Collapse Disorder in Spain. Study of causes and consequences",** has focused in the study of the implication of honey bee viruses in colony losses from a global perspective, considering both viral diagnosis and epidemiology of viruses in association with other factors which may have a potential effect on honey bee colonies. The work developed in this thesis has produced four scientific articles, two of them published and two of them under review in scientific journals. On one hand, these articles comprise molecular diagnosis and metagenomics for the detection of viruses in samples from colonies with symptoms compatible with CCD (objectives 1 and 2). On the other hand, regarding the peculiarities of viral infections in honey bees and the multifactorial origin of colony losses, viral diagnosis has been integrated in epidemiological studies where the influence of other factors in the pathological effect of viruses has been considered, including both internal and external factors. Thus, this thesis has studied the presence, load

and seasonality of honey bee viruses important for their potential pathogenicity in two Spanish regions, Andalucía and Madrid. The relationships between bee viruses and other factors such as beekeeping practices and presence of other pathogens have also been evaluated (objectives 3 and 4).

The **first objective** of this thesis was studying the potential relationship between Israeli acute paralysis virus (IAPV) and CCD in honey bee colonies in Spain. This virus was described as an indicator of CCD in USA in 2007, and its presence in Spain was detected in 2010. However, this objective quantifies for the first time and analyzes the potential implication of IAPV in CCD in Spain, selecting the region of Andalucía as study area. The frequency of IAPV in the sampled colonies, diagnosed by RT-PCR, was 13.5% and the average viral load $4.9x10^5$ GEC/bee, although loads between 10^2 and 10^7 GEC/bee were detected in the sampled colonies. However, no association between weak colonies and IAPV was observed and the highest IAPV loads were found both in healthy and weak colonies. These results suggest that high IAPV loads do not produce colony collapse by themselves, although further studies regarding histopathological analysis of individual honey bees would be interesting to assess the effect of these IAPV loads. Therefore, the origin of the IAPV isolate from Andalucía was studied using phylogenetic analysis, which revealed an association with sequences from France in a cluster different from the Spanish sample previously described in Valencia. Thus, these results indicate the presence of two different evolutionary lineages of IAPV with different origin in Spain.

The **second objective** of this thesis was analyzing the presence of unknown viruses potentially implicated in SDC using metagenomics. This objective arises due to the limitations of the diagnosis of honey bee viruses such as: the lack of antibodies, the lack of cell cultures, the usual presence of viruses in coinfections and the limitations of molecular diagnosis, which requires the previous knowledge of the viral sequence. Metagenomics is based on the high-throughput sequencing of viral genomics, overcoming these limitations and thus, offering an alternative and a unique opportunity to study viruses implicated in colony losses. Using this approach, the presence of viruses was analyzed in one sample from a colony with CCD in Navarra. Three viruses were coinfecting this sample: IAPV and, for the first time in Europe, aphid lethal paralysis virus (ALPV) and Lake Sinai virus (LSV), two viruses previously described in USA which require further studies about their pathogenicity in honey bees and their role in CCD. The phylogenetic analysis of IAPV revealed similarities with the isolate from Andalucía previously described and other sequences in France, probably due to the geographical proximity. This study also revealed

that honey bees can carry a plant virus, turnip ringspot virus (TuRSV), potentially serving as important vector organisms.

The **third objective** of this thesis was determining which beekeeping practices can influence in the effect of viral infections in colony health. Understanding the effect of beekeeping practices in colony health can help to its optimization as a strategy for the prevention of viral diseases that can lead to the colony collapse. An extensive sampling was performed in the region of Andalucía to characterize beekeeping practices and the presence and load of honey bee viruses (deformed wing virus (DWV), black queen cell virus (BQCV), acute bee paralysis virus (ABPV), IAPV, Kashmir virus (KBV), sacbrood virus (SBV) and chronic bee paralysis virus (CBPV)) in two samplings in spring-summer and autumn-winter. A slight deterioration of health status was appreciated in the autumn-winter, together with fungal disease and unspecific symptoms such as kleptoparasitism of neighboring colonies, although no viral symptoms were appreciated. The presence of bee viruses was common in both samplings, however only BQCV and DWV were detected in all provinces in both samplings, and only DWV showed increased frequency and load between samplings to a maximum of 10^7 GEC/bee and without presence of wing deformities. Trends regarding associations between beekeeping practices and honey bee viruses were observed for BQCV and DWV. Importantly, association between beekeeping practices and factors related to professional handling and lower virus presence was found. Thus, training courses in beekeeping practices are essential for maintaining an optimal health status of colonies.

The **fourth objective** of this thesis was to analyze the dynamics and interactions of honey bee viruses with other pathogens and their implication in colony weakening. To that end the presence of viruses (DWV, BQCV, ABPV, IAPV, KBV and SBV), one microsporidium (*Nosema ceranae*) and one parasite (*Varroa destructor*) was analyzed in samples from adult bees and brood, in spring-summer and summer-winter seasons in six commercial apiaries located in natural environments in Madrid. Although the presence of pathogens was frequent in spring-summer, prevalences and loads increased in summer-autumn together with the presence of overt symptomatology and colony weakening. Principal component analysis revealed that colonies from the same apiary have similar pathogen presence and load, and thus, characterization of the environment of colonies could be essential in the study of pathogen dynamics in honey bee colonies. Moreover, a positive correlation was found between BQCV and SBV loads in the two samplings, and between DWV and Varroa only in summer-autumn. Weakening of colonies was associated

with the presence of high DWV loads in summer-autumn, when overt symptoms were also observed. Therefore, study of adult and brood samples separately also revealed that brood samples had higher DWV load in this season, indicating that this is an interesting sample in the study of Varroa-DWV dynamics and implications in colony collapse. Finally, we first report the presence ABPV for the first time in Spain.

Thus, this thesis represents a major progress in the study of the implication of honey bee viruses in colony losses from a multidisciplinary perspective, combining diagnosis and epidemiology, in colonies of Spain, which has the highest census and honey productions in the European Union. The results obtained in this thesis will allow the implementation of control measures and surveillance which could help to minimize the effect of viruses in colonies, maintaining their health status.