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Epidemiological Survey of *Ascosphaera apis* in Small-Scale Migratory *Apis mellifera iberiensis* Colonies

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Abstract

Honey bee hives are moved yearly mainly for pollination, but also to take advantage of consecutive flowering events to get as many harvests of honey as possible and/or to find favorable sites for food sources and summer temperatures. Such movements may lead to pathogen spill-over with consequences on the honey bee health and finally on population decline. *Ascosphaera apis* is the causative agent of the chalkbrood disease, a pathology affecting honey bee larvae that significantly harms population growth and colony productivity. In this study, we detected the presence of *A. apis* in adult worker honey bees by PCR-amplification of the intergenic transcribed spacer (ITS1) of the ribosomal gene (rDNA). We first optimized the DNA extraction by testing different protocols in individual and pooled (colony level) adult honey bee samples. Subsequently, the presence of the fungus *A. apis* was assessed in both stationary and migratory colonies (subjected to small scale regional level movements) to determine the effect of migratory practices on the dispersal of this pathogen. Results confirmed a higher prevalence of *A. apis* in migratory apiaries when compared to stationary ones, indicating that migratory colonies are more likely to develop chalkbrood disease. Given these results, we suggest that beekeepers should be aware of the risks of pathogens spreading while moving beehives, even within a reduced geographic range.

Introduction

The benefits of safeguarding pollinators, especially the honey bee *Apis mellifera* as the most economically important pollinator insect, have been recently articulated (Potts et al., 2016). In this sense, sustainable hive management is among the strategies to avoid pollinator decline, as it has been shown that techniques such as migratory beekeeping increase oxidative stress levels in honey bees (Simone-Finstrom et al., 2016), and also favor the spread of pathogens (Cavigli et al., 2016).

Recently, attention has been paid to the impact of transporting beehives on spreading diseases (Ahn et al., 2012; Zhu et al., 2014; Simone-Finstrom et al., 2016; Traynor et al., 2016; Guimarães-Cestaro et al., 2017). While in North America honey bee hives are moved yearly for pollination of monocultures, in Spain beehives are mainly moved to take advantage of consecutive flowering events (to get as many harvests of honey as possible) and to find favorable sites for food sources and summer temperatures. Migratory beekeeping is an extended practice in this country. There are 2.7 million beehives (data from 2015; REGA 2016), of which 80% are moved by professional beekeepers (a beekeeper is considered to be professional when he/she manages more than 150 beehives per year; mean is 406 beehives per beekeeper). Although this practice usually leads to increased honey production, it can imply higher costs due to transportation and colony losses that may counteract the actual economic benefits.

Pathogen dissemination has been cited as one of the main causes of the severe decline of honey bee populations (Neumann & Carrek, 2010). Among pathogens, fungi might play an important role since spores can resist up to 15 years (Gilliam, 1986). *Ascosphaera apis*, the causative agent of

chalkbrood disease in honey bee larvae, can survive one year in honey and two in pollen (Flores et al., 2005a, b). This disease is found worldwide, is typically common during spring (Aronstein & Murray, 2010) and causes significant harm to population growth and colony productivity in weak colonies through a loss of honey bee workforce (Evison, 2015); however, those colonies that grow stronger over the summer can fully recover (Evison & Jensen, 2018) and often show no symptoms of the disease. In these cases, diagnostic techniques such as molecular assays are needed to detect the pathogen. *A. apis* has been shown to be difficult to identify due to the lack of distinctive morphological features and the requirement of a special medium and growth conditions (Jensen et al., 2013 but see Chorbinski & Rypula, 2003). The first biochemical method for identifying this fungus was based on isozyme analysis (Gilliam & Lorenz, 1993; Chorbinski, 2003). Later, internal spacers (ITS1, ITS2) of ribosomal rDNA have been shown to be particularly useful in elucidating the relationships between closely related species of *Ascosphaera* (Anderson et al., 1998; Chorbinski, 2004; Borum & Ulgen, 2008). These analyses facilitated the design of primers specific for the ITS1-5.8S-ITS2 rDNA region for each *Ascosphaera* species (James & Skinner, 2005; Murray et al., 2005; Yoshiyama & Kimura 2011). More recent diagnostic methods rely upon information from the *Ascosphaera* genome (Qin et al., 2006) depicting polymorphic loci that can be used to differentiate haplotypes in *A. apis* (Jensen et al., 2012).

As Aronstein and Murray (2010) noted, 'the migratory nature of commercial beekeeping in North America and Australia is probably the most important factor contributing to the rapid spread of chalkbrood disease within these two continents'. It can be then hypothesized that migratory hives have a higher incidence of *A. apis* due to the close contact among hives while transporting them, and the intrinsic management of this beekeeping practice. To test this hypothesis, we first implemented the molecular characterization of the causal agent of chalkbrood in adult worker honey bees. Then we searched for *A. apis* in symptomatic and asymptomatic colonies of both stationary and migratory apiaries from Murcia, a south-eastern Mediterranean Spanish province. Murcia has 93,954 beehives grouped in 485 apiaries of which 420 usually practice migratory beekeeping, and transportation occurs at different scales, nationally (around 700 km) to regionally (80 km). This study investigates a possible correlation between the prevalence of *A. apis* in honey bee colonies and the practice of small-scale migratory beekeeping.

Material and Methods

Samples (20 to 25 honey bee workers) were taken from the inner frames of 46 beehives of *A. m. iberiensis* during spring (2015), when the incidence of *A. apis* is presumably higher (Flores et al., 1996; Borum & Ulgen, 2008). From these 46 beehives, 21 were stationary (8 belonging to an apiary at the locality of Miravetes and 13 from our research apiary at the, University of Murcia, Espinardo Campus) i. e. those colonies are not moved from the apiary in any moment of the year; the remaining 25 beehives were moved yearly around 60 km from their original locations (10 from Loma Ancha, 6 from El Llano and 9 from Pantano Puentes migratory apiaries) (Figure 1 and Table 1).

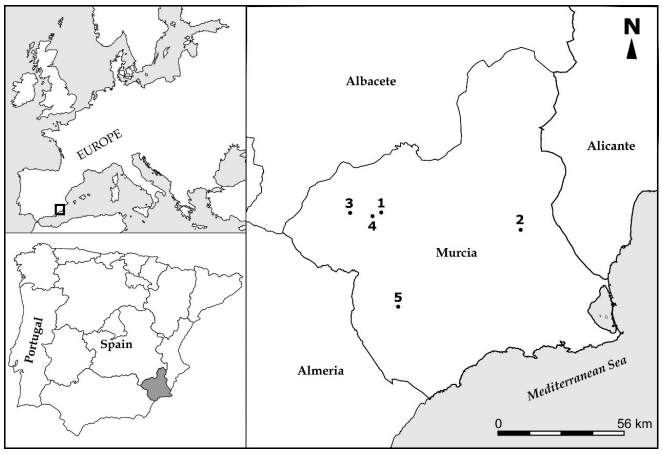

Fig 1. Location of the apiaries sampled in Murcia (SE Spain). Stationary hives were located in 1 (Miravetes) and 2 (Espinardo Campus), while migratory hives were located in 3 (Loma Ancha), 4 (El Llano) and 5 (Pantano Puentes).

Different protocols were tested to optimize DNA extraction and PCR amplification of the fungus *A. apis*. DNA was first extracted at the individual level using two legs of each worker honey bee following either the Ivanova et al. (2006) or Chelex® methods (Evans et al., 2013). At the colony level, two tests were performed: one consisted on a macerate of five workers per colony, from which DNA was extracted by following a modified protocol from Martín-Hernández et al. (2007), and the second consisted of a pool of ten legs (two from each of the five workers), from which DNA was extracted with a modified Chelex® method by adding 200 µl of 5% Chelex® and 10 µl of proteinase K (10 mg/ml). DNA extraction yields obtained from each protocol were assessed by using NanoDrop 1000 Spectrophotometer (NanoDrop Technologies).

The rDNA region of *A. apis* was amplified using the primers 3F1F: 5'-TGT CTG TGC GGC TAG GTG-3' y AscoAII2R: 5'-GAW CAC GAC GCC GTC ACT-3' (James & Skinner, 2005) in a thermocycler PTC 100 (MJ Research), by adding 2 µl of DNA of each sample to a final reaction volume of 15 µl. The program cycle used was as follows: denaturation for 3 min at 94 ºC; 36 cycles of 30 s at 96 ºC, 30 s annealing at 55 ºC and extension for 30 s at 68 ºC; then a final elongation step of 20 min at 65 ºC (modified from Yoshiyama & Kimura, 2011). Positive (DNA extracted directly from the fungus isolated from a mummy honey bee worker) and negative controls were included in each reaction. Amplicon size was confirmed after electrophoretic separation on 1.5% agarose gels.

Correlation between the variables "stationary" and "migratory" and "presence of *A. apis*" was analyzed using the Chi-squared test, the Fisher exact test, and Cramer's V for contingency tables (PAST v3.17 program, Hammer et al., 2001).

Results

DNA concentration obtained from the different tested extraction protocols was appropriate for PCR amplification in all cases, yielding from 9 to 115 ng/µl of DNA. The highest extraction yields were obtained with the Chelex® protocol at both individual and colony levels. Therefore, we adopted the protocol adapted to pools of legs of honey bee workers for processing all subsequent samples at the colony level.

To set up the amplification reaction of the ITS region of *A. apis* we extracted DNA from honey workers selected from a colony with chalkbrood symptoms (Figure 2) and directly from the fungus as a positive control. When using 55 ºC of annealing temperature, amplicons of the expected size (442 base pairs) were obtained.

Table 1. Geographical information about the apiaries included in the study and percentage of colonies in which *A. apis* was found in stationary and migratory apiaries. Number in brackets refer to the apiaries in locations from Fig 1.

	Stationary		Migratory		
	Miravetes (1)	Campus of Espinardo (2)	Loma Ancha (3)	El Llano (4)	Pantano Puentes (5)
GPS data Latitude/ Longitude	38º5'54"/1º53'45"	40°1'34.71"/3°10'33.82"	38º5'50"/2º0'058.8"	38º6'11.8"/1º51'58.4"	37º43'54.3"/1º47'41.5"
No. of hives	8	13	10	6	9
% of infected colonies	50	76.92	100	100	100

The next step was to detect *A. apis* in the migratory and stationary apiaries at the colony level. The fungus *A. apis* was detected in all 25 colonies belonging to the three studied migratory apiaries. In the case of stationary apiaries, 50% of the colonies from Miravetes and 77% of the colonies from Espinardo Campus were positive for the presence of *A. apis* (Table 1). In total, 100% of migratory colonies and 67% of stationary colonies were positive for the fungus. A significant difference ($\chi^2 = 23.95$, df = 1 and $p < 0.00$; Fischer exact test < 0.001) between the presence of *A. apis* and the beekeeping management of the colonies was observed. Additionally, Cramer's V (0.722) showed that beekeeping management was strongly associated with the presence of *A. apis*, suggesting that these factors (stationary or migratory colonies) affected the probability of occurrence of chalkbrood disease.

Fig 2. Chalkbrood at the entrance of the beehive, indicating the development of the disease.

Discussion

One of the drivers of pollinator decline at the global level is the dispersion of pathogens and parasites; therefore, the study of the prevalence of these organisms is of increasing interest. In this study, we detected the fungus *A. apis* by extracting DNA with the Chelex method directly from adult worker honey bees sampled from brood combs. This extraction method is widely used in population genetic studies of the honey bee (Evans et al., 2013), and it has also proven to be useful for detecting the fungus both at the individual and colony levels. This is particularly relevant in the case of social insects, as population studies include large number of samples, hence the need for cheap, fast, and effective molecular tools to monitor individual or colony health and exposures.

In our study we have set up the detection of *A. apis* spores in adult honey bees to further increase the knowledge of the impact of beekeeping techniques as migration of hives, on the spread of this fungus and its associated disease. In this sense, although some condition (temperature, habitat) differences exist in the stationary apiaries, the percentage of colonies positive for *A. apis* in the stationary apiary at the Espinardo Campus was higher (77%) than that found in the apiary of a private beekeeper (50%). Colonies at the University are subjected to continuous management and experimentation procedures what may be considered as stress factors. It is possible that the higher proportion of infected colonies is the result of fungus transmission through the use of contaminated beekeeper material, because spores can accumulate in all parts of the beehives and in all beehive products (e.g. foundation wax, stored pollen and honey), and remain viable for at least 15 years (Gilliam, 1986). It is therefore recommended to carry out management procedures with clean instruments, to minimize the dispersion of the fungus spores and the possible

development of the disease. Likewise, combs should be replaced yearly to avoid transfer of combs between potentially infected beehives (Flores et al., 2005a, b).

The results of the statistical analysis confirm the initial hypothesis about the effects of migratory beekeeping on the dispersion of the fungus *A. apis*. The percentage of detection of the pathogen was significantly lower in stationary than in migratory colonies (50-77% stationary, 100% migratory). The intensive management of migratory colonies makes them prone not only to a higher prevalence of the fungus, but also to a rapid dispersal of spores across distant areas; both transport stress and spore contamination may explain the high incidence of chalkbrood disease in moved colonies mentioned by Aronstein and Murray (2010). During migratory events, apiaries dedicated to economic exploitation need constant supervision and management, including handling with the same equipment, which increases the possibility of contagion from one colony to another. In addition, because of continuous management to produce more honey, colonies may suffer a nutritional deficiency that increases the likelihood of disease development, since shortage of essential amino acids will impair the correct functioning of the immune system (Di Pasquale et al., 2013). Other undesirable factors that affect migratory colonies in relation to a higher incidence of *A. apis* fungus could be stress upon honey bees resulting from truck noise and vibrations, changing temperatures among the visited sites (Simone-Finstrom et al., 2016) and a higher drift of workers during migratory operations (Fries & Camazine, 2001). Similar results to those obtained here have been observed in studies in Turkey including other pathogens as *Nosema* spp (Tokzar et al., 2015) although this microsporidium has a different biology. These authors noted a higher prevalence of *Nosema ceranae* in migratory colonies than in stationary colonies. However, any significant relationship between hive management and pathogen dispersal was found in other studies in which the prevalence of pathogens and parasites related to the decline of honey bee colonies (for instance *Paenibacillus larvae*, *Varroa destructor*, *N. apis* and *N. ceranae*) in stationary and migratory apiaries in Brazil has been evaluated (Guimarães-Cestaro et al., 2017), suggesting that there may be also other factors that influence the spread of pathogens such as environment stressors as climate (van Engelsdorp et al., 2013), and even the honey bee subspecies or evolutionary lineage that could show different resistance traits (Jara et al., 2012).

A. apis spores geminate in the anaerobic environment of the closed hindgut of two–four day old larvae after their activation by CO_2 (Bamford & Heath, 1989), therefore adult honey bees are not susceptible to chalkbrood disease, but they can transmit the pathogen within and between beehives because fungal spores carried by foraging honey bees are passed to nursing honey bees that then feed larvae with contaminated food (Aronstein & Murray, 2010). This possibility should be taken in consideration by beekeepers, as one of the drivers of pollinator decline at the global level is the dispersion of pathogens and parasites. In conclusion, beekeepers should find a balance between economic benefits of migrating colonies (even at a small-scale) for a continuous honey harvesting and the impact of spreading pathogens and stressing the colonies that increases the incidence of diseases.

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