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Article

Ascospherosis in honey bees and its relationship to environmental factors in Jalisco, Mexico

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Abstract:

Ascospherosis or chalkbrood is an infectious disease of honey bees (*Apis mellifera*) caused by the fungus *Ascosphaera apis* that results in the death of larvae. In severe cases it can cause a decrease

of the population of adult bees and in the productivity of the colony. This is the first study performed in Mexico with the objective of determining the prevalence of *A. apis* in honeybee colonies in a beekeeping region. It was carried out in nine municipalities of southern Jalisco, distributed in two climatic zones (sub-humid and subhumid temperate warm), and the relationship of the fungus with factors such as height above sea level, precipitations and temperature was analyzed. Samples of bees were collected from 365 breeding colonies, of which 74.1 % were proven chalkbrood-positive by microscopic analysis. 75.6 % of the colonies in the warm area and 72.2 % of those of the temperate area were *A. apis*-positive and were not significantly different in terms of the prevalence of chalkbrood ($P>0.05$). The logistic regression analysis indicated that the increase in rainfall also significantly increases the number of colonies infected with *A. apis* (odds= 3.53; $P<0.01$). There was a significant correlation between the rainfall and the proportion of ascospherosis-positive cases ($r= 0.87$, $P= 0.003$). The other studied factors had no significant relationship with cases of ascospherosis. Due to the high percentages of colonies infected with *A. apis* found in this study, further research should be considered in order to determine the causes of the high prevalence of this fungus in honey bee colonies in the state of Jalisco.

Key words: *Ascosphaera apis*, *Apis mellifera*, Environmental effects, Jalisco.

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Introduction

Ascospherosis or chalkbrood is a fungal disease of honeybees (*Apis mellifera* L.) caused by the fungus *Ascosphaera apis*[1]. The fungus that causes chalkbrood affects mainly the larvae but also the pupae of bees. The two main routes of infection are digestive, through consumption of food contaminated with spores of the fungus, or else, the cuticle of the offspring, through the germinative tube coming out of the spores. Both forms of infection produce mycelia that penetrate the body of the larvae, causing their death and giving them the characteristic of mummies[2]. There are predisposing factors that favor the occurrence of ascospherosis outbreaks, such as excess moisture inside the hive, low temperatures or excessive manipulation of the colonies[3]. Empirical observations suggest that chalkbrood increases with the stress caused by the frequent transportation of hives in order to pollinate agricultural crops, as a consequence of the exposure to the pesticides used on the pollination sites, and due to the new pathogens that affect bees. All of these factors depress the immune system of the bees and favor the development of other pathogens, including

A. apis[4]. Mortality from chalkbrood in the offspring of bees is generally low, but there are times when it exceeds 30 %[5]. The disease has been reported in almost all countries in the world, including Mexico[5-9]. It is important to highlight that the prevalence of ascospherosis in certain countries increased considerably, to the extent that it has been considered a threat almost as serious as infestation with *Varroa destructor*[10].

Ascospherosis has been little studied in Mexico, where it has not been given the required importance. However, in Yucatán, Medina and Mejía[11] found an association between ascospherosis and the collapse of bee colonies. Other than isolated studies carried out in Yucatán, it may be said that there are no data that allow knowing how prevalent and generalized chalkbrood is in Mexico, as no epizootiological study has been carried out to determine its prevalence in the country or in a particular state of the country. Furthermore, the situation of ascospherosis and the relationships that it may have to geographical and climate variables in apiaries of Mexico are unknown; therefore, the objective of the present study was to determine the presence of the disease in honey bee colonies and its relationship with geographic and climate variables in the municipalities of the south-southeast region of the state of Jalisco, Mexico.

Material and methods

The study was carried out in nine municipalities located in two climate areas considered to be temperate and warm (temperate subhumid and warm subhumid climate, respectively) in order to determine the prevalence of *A. apis* and it there is a relationship between the climate and the prevalence of the fungus in honey bee colonies. The municipalities are located in western Mexico (19º 24', 21º 14' N; 101º 59', 104º 5' W). The Gómez Farías, Zapotlán el Grande, Tapalpa and Unión de Guadalupe municipalities have a temperate subhumid climate, and the Tecalitlán, Tamazula, Zacoalco de Torres, Sayula and Cocula municipalities have a warm subhumid climate[12]. The municipalities with a warm subhumid climate have a mean annual temperature of 21 °C. This climate does not exhibit a clear-cut winter temperature change[13]. Rainfalls occur from June to September, with a mean precipitation of 801 mm, and the average altitude is 1,495 m asl[12]. Municipalities with a temperate subhumid climate have a well-defined winter temperature change; the mean annual temperature is 14.4 °C, and the mean annual precipitation is 1,117 mm; the average altitude is 1,711 m asl[13]. Samples were collected from 365 honey bee colonies in 145 apiaries in both geographical areas. The sample size was estimated based on 4,950 beehives in the selected regions of the state of Jalisco. 365 (13.56 %) of these beehives were selected for analysis. The calculation was made using the formula of transversal studies and a disproportionate stratified random sample with a 95 % confidence interval and an 80 % statistical power. The result was 21 samples per municipality including 15% due to losses, for a total of 160 beehives. However,

the decision was made to take a total of 365 beehives to obtain a greater accuracy in the analysis (Table 1).

Table 1: Number of apiaries and colonies sampled for diagnose of *Acosphaera apis* by municipality and climate area in southern Jalisco

Municipality	Apiaries	Colonies	Climate area
Cocula	17	41	Warm subhumid
Sayula	21	52	Warm subhumid
Tamazula	36	93	Warm subhumid
Tecalitlán	21	54	Warm subhumid
Zacoalco de Torres	14	39	Warm subhumid
Gómez Farías	6	15	Temperate subhumid
Tapalpa	12	37	Temperate subhumid
Unión de Guadalupe	8	20	Temperate subhumid
Zapotlán el Grande	10	14	Temperate subhumid
Total	145	365	

Samples of the honeycomb were obtained from the central frames of the brood chamber where the open brood of the bees was located, and in certain cases signs of ascospherosis (mummification of the brood) were observed. A 10 x 10 cm piece of honeycomb of each assessed colony was cut and wrapped in a paper sheet and kept in a cardboard box in order to be transported to the laboratory where the diagnose was carried out. The altitude of each apiary was recorded using a GPS (Sportrack-color, Magellan, USA), while the data of the environmental temperature (ºC) and the rainfall (mm), were taken from the records of the National Commission of Water for each municipality[14].

The brood contained in the samples of honeycombs were processed at the Laboratory of Microbiology of the University Center of the South (CUSur) of the University of Guadalajara, where lactophenol cotton blue was utilized to diagnose the fungi. The utilized slides were cleaned with an ethyl ether solution; once they were dry, a drop of physiological salt solution was added, in which a small fragment of a larva suspected to be infected with chalkbrood fungus was placed. The larval sample was extended with a toothpick until it absorbed the drop of water; then a drop of lactophenol cotton blue was deposited on the larva and covered with a slide. Subsequently, *A. apis* mycelia and sporocysts were sought under the dry weak objective of a microscope (40 X). The presence of *A. apis* was determined based on the finding of sporocysts that are typical of the fungus

in the analyzed samples[15], as well as on the percentage of beehives with ascospherosis in each of the municipalities.

In the statistical analysis, the response variable was the number of colonies of bees infected with the *A. apis* fungus between different municipalities. The predictors (explanatory variables) included in the analysis were: environmental temperature, rainfall, and height above the sea level. The explanatory variables were standardized in order to reduce multicolinearity issues. The statistical analysis was performed using the following techniques. The proportions of infected colonies between municipalities and between areas were compared using the Chi-square tests with Bonferroni adjustments for identifying significant differences. A multiple logistic regression analysis was performed according to the criteria of Hosmer and Lemeshow[16] in order to identify factors associated with the fungal infection; this analysis allowed calculating the ratio of likelihood (odds) of finding ascospherosis-positive colonies and its corresponding 95% confidence interval (CI). A Pearson's correlation was also carried out between the proportion of ascosphorosis-positive cases and the factors. The statistical analyses were performed using the R package, version 3.3.2[17].

Results

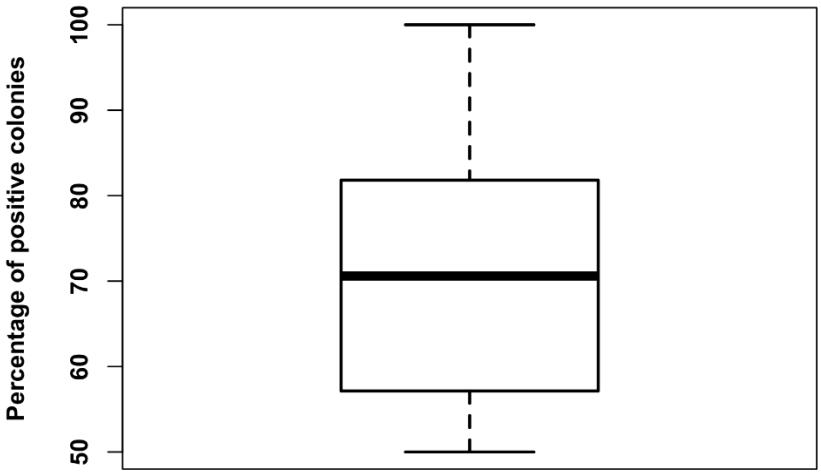
Of the 365 assessed samples, 74.1 % were found to be *A. apis*-positive. The municipalities with the highest percentages of positive samples were Gómez Farías and Zacoalco de Torres, with 93.3 and 92.3 %, respectively, while those that exhibited lowest percentages, were Zapotlán el Grande, Unión de Guadalupe and Tecalitlán, with 57.1, 60.0 and 61.1 %, respectively (Table 2).

Table 2: Mean percentages of *Ascosphaera apis*-positive honey bee colonies in nine municipalities of two climate areas of southern Jalisco

Municipality	Mean percentage	Climate area
Cocula	82.93	Warm subhumid
Sayula	73.08	Warm subhumid
Zacoalco	92.31	Warm subhumid
Tamazula	68.82	Warm subhumid
Tecalitlán	61.11	Warm subhumid
Tapalpa	78.38	Temperate subhumid
Gómez Farías	93.33	Temperate subhumid
Unión de Gpe.	60.00	Temperate subhumid
Zapotlán el Grande	57.14	Temperate subhumid
Total	74.10	

Apiaries with 100 % *A. apis*-positive colonies were found in the municipalities of Gómez Farías, Cocula, Sayula, Tamazula, Tapalpa, Tecalitlán, Zapotlán el Grande and Zacoalco de Torres; however, in Unión de Guadalupe, the highest percentage of positive colonies in an apiary was 60 %. The distribution of the percentage of colonies infected among the nine municipalities was examined by means of a box plot (Figure 1). The figure shows that the median of the percentage of infected colonies was 71 %. Half of all the sampled colonies exhibited ascospherosis prevalence values ranging between 57 and 82 %.

Figure 1: Box plot depicting the distribution of the percentage of bee colonies infected with the fungus *Ascophaera apis*



The values shown here include the minimum percentage, in quartile 1 (25 % of the data); the mean (50 % of the data); the maximum percentage, in quartile 3 (75 % of the data), and the range between quartiles.

A total of 36 comparisons between the proportions of bee colonies infected with *A. apis* in the nine municipalities. When the critical area was adjusted for multiple comparisons, the differences between proportions were not significant (P>0,05). As for the areas, the results show that the prevalence of ascospherosis between the warm (75.6 %) and the temperate (72.2 %) areas did not differ significantly (x^2= 0.86, n= 209, P= 0.35). The results of the logistical regression analysis are shown in Table 3. The equation of the complete logistic regression model (the model including all the predictors) was the following:

$$\log \text{odds} = 1.04141 - 0.03434\ \text{Temperature} + 1.2188\ \text{Precipitation} - 0.02706\ \text{Height above the sea level}$$

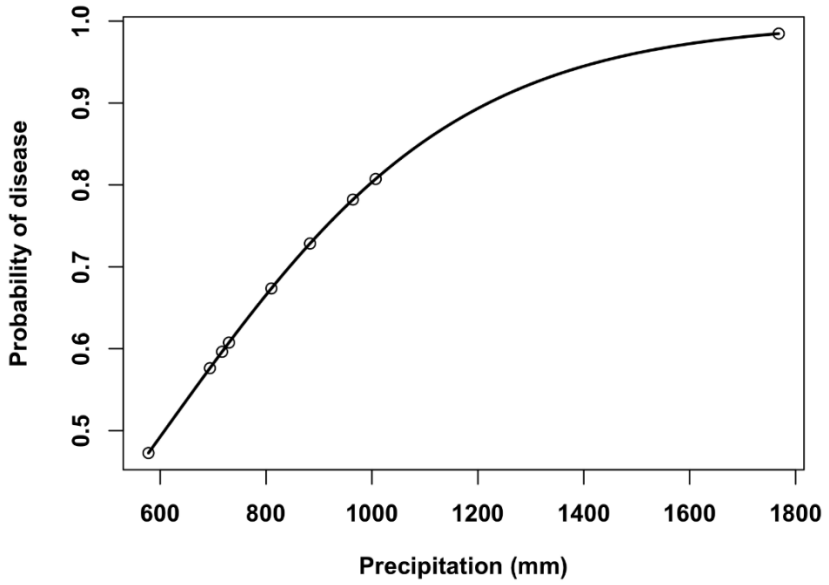
Table 3: Predictors of infection of honey bees by the fungus *Ascosphaera apis* in the logistic regression analysis (complete model)

Variables	Probability (Odds)	95 % CI	*p*
Temperature	*0.97	(0.69 - 1.37)	0.84
Precipitation	*3.38	(1.73 - 7.48)	<0.01
Height above the sea level	*0.97	(0.64 - 1.50)	0.89

**Ascosphaera apis* colonies.

The analysis showed that precipitation was significantly associated to infection of bee colonies with the fungus. The increase in the rainfall also increased the number of colonies infected with *A. apis* significantly (odds= 3.35; *P*<0.01; Figure 2). Likewise, a significant correlation was found between the rainfalls and the proportion of ascospherosis-positive cases (r= 0.87, n= 9, *P*= 0.003). The other studied factors had no significant relationship with cases of chalkbrood.

Figure 2: Relationship between the rainfalls and the proportion of positive cases of infection by the fungus *Ascosphaera apis* in nine municipalities evaluated in Jalisco, Mexico



Discussion

This is the first study carried out in Mexico with the purpose of determining the prevalence of *A. apis* in honey bee colonies in several beekeeping regions of a state. In a similar study carried out in the semi-desertic region of the Highlands of Jalisco, Mexico, during the months of July to October (the rainy season), in which 42 colonies distributed along 12 km only within this region were monitored, 67.1 % of the colonies were found to be *A. apis*-positive[18]. The study of the semi-desertic region of the Highlands of Jalisco is based on a very small sample (42 colonies) of a restricted area of the state of Jalisco, whereas the present study included 365 colonies in the two regions with the largest production of honey in the state of Jalisco. However, it is worth noting that, although the study of the Highlands of Jalisco was performed during the rainy season, the percentage of positive samples was similar to the percentage found in the present study, performed during the dry season (74.1 %). This reinforces the hypothesis that the prevalence of chalkbrood in the bee colonies of the state of Jalisco is high at different times of the year. The environmental temperature influences the temperature and moisture of the beehive, but the bees create their own inner microclimate of moisture and temperature[19]; therefore, it is difficult to determine the impact of the external environmental climate during the rainy season and during the dry season. However, other researches mention that a reduction of the inner temperature of the colony from 35 to 30 °C increases the prevalence of *A. apis*[20].

In regard to the moisture, other studies agree with the findings of this study in the sense that the rainfalls are related to a high prevalence of chalkbrood. For example, in a recent research, relative moistures of 85 to 90 % and inner temperatures of 25 to 30 °C in *A. mellifer*a colonies have been determined to favor the occurrence and proliferation of *A. apis* spores[21]. On the other hand, colonies that keep the brood nest relatively dry and warm (> 35 °C) significantly limit infection by *A. apis*. Bramford and Heath[22] also determined that the temperature and the internal moisture of the brood nest are factors that predispose for the presence of *A. apis* in the colony. These authors assessed the cooling of the colony at 25 °C, which resulted in 95 % of chalkbrood mummies, while mummification diminished to 43 % with 30 °C, and to 29 % with 35 °C. As for the relative moisture, when it surpassed 85 % at a temperature of 30 °C, mummification increased by 7.75 % in relation only to the effect of the temperature at 30 °C. Likewise, with 68 % moisture at a temperature of 30 °C, the mummification of larvae of the colony increased only by 0.95 %.

Clearly, low temperatures and a high level of moisture favor the occurrence of ascospherosis. Other researchers have found that other factors, such as stamped wax and pollen. contribute to the transmission and development of the chalkbrood disease in bee colonies[5,23]. Also, a reduction in the proportion of adult bees to the brood contributes to the prevalence of the disease[24]. Some of

these factors may be contributing to the high prevalence of chalkbrood in Jalisco. However, only further studies will make it possible to determine whether these or other factors are responsible for this high prevalence of the disease.

Conclusions and implications

74.1 % of the 365 assessed samples were found to be *A. apis*-positive; this constitutes a high prevalence of the fungus in bee colonies of southern Jalisco. The prevalence of *A. apis* was particularly high in certain municipalities, where the average of positive samples reached over 90 %, and there were no differences in prevalence between the areas. The climate factor which had a significant association with the presence of *A. apis* and which most probably promoted a higher occurrence of the fungus in the bee colonies was rainfall. The altitude and environmental temperature had no significant effects on the prevalence of *A. apis* in this study. Due to the high percentages of *A. apis* in the assessed colonies, further studies must be considered in order to determine the causes (other than rainfall) of the high prevalence of *A. apis* in the bee colonies of southern Jalisco.

Acknowledgments and conflicts of interest

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