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Evaluation of large-scale dissemination of *Nosema ceranae* spores by European bee-eaters *Merops apiaster*

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Summary

Identification of transmission routes and of factors affecting the spatial positions of pathogens, hosts and vectors is basic to an adequate disease management. *Nosema ceranae* is a Microsporidian recently described as a parasite of *Apis mellifera* honeybees and is currently considered the aetiological agent of an emergent illness named nosemosis type C. In this article we evaluate the role of a bird species, the European bee-eater, *Merops apiaster*, as a large-scale dispersive agent of *N. ceranae*. We found a high prevalence of viable spores of *N. ceranae* in pellets regurgitated by bee-eaters in different locations in the Iberian Peninsula, Central Europe and central Asia. In contrast, spores of *Nosema apis*, considered till recently the most common microsporidium infecting honeybees, were detected in a single locality and *Nosema bombi* spores were not noticed. Since non-viable spores were also found in bee-eater nests from different locations, this bird species could also reduce the fraction of infected insects by withdrawing pathogens from the colonies. We conclude that bee-eater mobility and migration may have played an important role in the transmission of the pathogen *N. ceranae*.

Introduction

Pathogens use many different modes to disperse from an infected to an uninfected host but, in most cases, the probability of transmission declines dramatically with distance from an infected host. As a consequence, identification of transmission routes and knowledge of the factors affecting the spatial positions of pathogens, hosts and vectors is of great epidemiological importance for an adequate disease management (Ostfeld *et al*., 2005). It is well established that free-living birds have the potential to disperse certain pathogenic microorganism (see review in Hubálek, 2004), as it has been recently shown for the West Nile virus in North America (see, for instance, Rappole *et al*., 2000; Malkinson *et al*., 2002; Komar *et al.,* 2003) and for influenza A viruses (Olsen *et al*., 2006). Yet, the extent to which migratory birds transmit pathogens among locations and the factors affecting the efficiency of such dispersal are poorly known.

Over the past years, a decline in pollinator populations has been detected all over the world (Williams, 2005; FAO, 2008) and a surprisingly high loss of honeybees has devastated a large number of honeybee colonies worldwide (Stokstad, 2007). In the USA Colony Collapse Disorder (CCD) is the term applied to denominate the drastic decrease of Western honeybee colonies in North America. In Europe this phenomenon is named Depopulation Syndrome or Colony loss (Higes *et al*., 2005; see COLOSS Workshop, 2009). The consequences of such loss are economically and ecologically significant because pollination, in which wild and domesticated bees play a major role, is a critical process in both human-managed and natural terrestrial ecosystems (Plischuk *et al*., 2009).

To date, no consensus has been reached regarding the origin and symptoms of the phenomenon. Several factors, like changes in the use of agricultural land (Williams, 2005; Naug, 2009), environmental change-related stresses (e.g. malnutrition and pesticides, Sharpe and Heyden, 2009) or pathogens (e.g. Higes *et al*., 2005; 2006; Cox-Foster *et al.,* 2007; Otti and Schmid-Hempel, 2007; Stokstad, 2007; Anderson and East, 2008; Blanchard *et al*., 2008; Plischuk *et al*., 2008; Highfield *et al*., 2009), have been suggested as likely causes of bee colony losses. Even though a host of pathogens can be implicated in the decline of bees (Cantwell, 1974), the endoparasitic microsporidian *Nosema ceranae* is one of the major suspects of the recent honeybee colony losses

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(COLOSS Workshop, 2009; Higes *et al*., 2009a; 2010a,b; Korpela, 2009). Nosemosis is a common worldwide disease of adult honeybees caused by microsporidia. Two species of microsporidia have been described to infect *Apis mellifera*: *Nosema apis* and *N. ceranae*. *Nosema apis* infecting *A. mellifera* was described more than 100 years ago. However, *N. ceranae*, a microsporidium of the Asian bee *Apis cerana*, seems to have jumped from its original host to *A. mellifera* (Higes *et al*., 2006; Huang *et al*., 2007) and it is now frequently detected worldwide (see Higes *et al*., 2010a). Higes and colleagues (2008a; 2009b) showed that natural *N. ceranae* infection can cause the sudden collapse of bee colonies. Currently, *N. ceranae* is considered the aetiological agent of an emergent and important illness of *A. mellifera* named now nosemosis type C (COLOSS Workshop, 2009; Higes *et al*., 2010a). For bumblebees another highly pathogenic species of microsporidian, *Nosema bombi*, has been described (Fantham and Porter, 1914; Fries *et al*., 2001) but this pathogen has not been recorded infecting honeybees until now.

Due to the short time since the colonization of *A. mellifera* by *N. ceranae,* scarce epidemiological data are available and information about modes of transmission is urgently needed. The rapid, long-distance dispersal of *N. ceranae* has been attributed to several, non-mutually exclusive factors: (i) the transport of infected honeybees by professional or hobbyist beekeepers (Klee *et al*., 2007), (ii) transmission by means of infected hive structures (Van der Zee, 2009), (iii) via corbicular pollen, where viable spores of *N. ceranae* have been recorded (Higes *et al*., 2007), and (iv) via commercial royal jelly (Cox-Foxter *et al.,* 2007). Recently, Higes and colleagues (2008b) demonstrated the occurrence of viable spores of *N. ceranae* into the regurgitated pellets of European bee-eaters (*Merops apiaster*) in central Spain. Higes and colleagues (2008b; 2009a) suggested that pellets can act as fomites of infective spores and that the flying behaviour of bee-eaters could spread *N. ceranae* over long distances.

The European bee-eater (family Meropidae) is a migrant, insectivorous bird whose breeding area extends along southern Europe, northwest Africa, Mediterranean islands, and countries of the northern Mediterranean east to Pakistan, northern India and Afghanistan. A few birds are found in South Africa. It also occasionally breeds in northwest Europe. Large numbers of European bee-eaters migrate seasonally between breeding areas in Europe and Asia and their wintering grounds in tropical Africa and western India (Cramp, 1985; Fry, 2001). Bee-eaters feed on a wide range of insects but analyses invariably show preponderance of Hymenoptera (bees, bumble bees and wasps). The proportion of the diet made up by bees and wasps averages 70% (Fry, 2001). Of these, honeybees can comprise a large part – as much as 89% – of the diet (Cramp, 1985; Fry, 2001). After ingestion, the indigestible part of insects is compressed by the gizzard into a pellet that will be eventually regurgitated. Bee-eaters usually hunt within 1 km of their nest, but can forage up to 7 km away from the colony (Cramp, 1985). Bee-eaters nest in cavities at end of deep burrows. Nests are seldom reused but breeding colonies are usually re-occupied for several years (Cramp, 1985). Adult birds feed the nestlings in the nest where arthropod remains accumulate throughout the breeding season. A successful nest may have a layer of up to 2–3 cm of detritus and insect remains (Cramp, 1985), many of them being *A. mellifera* remnants (see, for instance, Fulton and Rose, 2007).

In this article we explore the importance of the European bee-eater in the transmission of *N. ceranae* at a large, continental scale. Moreover, we take advantage of the likely disseminating role of this bird to evaluate the relative prevalence of *N. ceranae, N. apis* and *N. bombi.* To meet these aims we monitored the occurrence of viable spores of these microsporidia in bee-eater pellets and nest remains from different colonies from southwest Europe, central Europe and central Asia.

Results and discussion

Here we describe the role of a bird species, the European bee-eater, as a large-scale dispersive agent of *N. ceranae*. Spores of this microsporidium were detected both by phase-contrast microscopy (PCM) and by polymerase chain reaction (PCR) methods in pellets from all sampled locations (Table 1), ranging from south-east and western Spain to central Asia (Fig. 1). This strongly suggests that *M. apiaster* is very likely to play a major role in the dissemination of *N. ceranae*. PCR analyses showed that spores of *N. ceranae* were highly prevalent in regurgitated pellets for the localities sampled, the lowest prevalence being found in the colonies from western Spain (range 33–50%) (Table 1). In contrast, PCM and PCR evidenced the occurrence of *N. apis* spores in a single locality, in western Spain, in a pellet where *N. ceranae* spores were also found. Spores of *N. bombi* were not detected in any sample.

The viability of *N. ceranae* spores was very high, averaging 93.3% (SE = 0.72, range: 88.3–98.3%). We found no differences in viability of spores among countries (Kruskal–Wallis test: $H_{3,20} = 5.05$, $P = 0.17$).

With regard to nest material samples, no spore was detected by PCR methods but 63.3% of the samples were positive to microsporidia-like spores by PCM methods (Table 2). In fact, the locations where no spores were detected by PCM (placed in south and central Spain) were the ones with the oldest samples (collected in 2002).

Table 1. Spore detection was performed by following a modification of the method described by Higes and colleagues (2008b).

Location	No. of pellets	Date	*Nosema bombi* [Viability]	*Nosema apis* [Viability]	*Nosema ceranae* [Viability]
Spain (Almería) 37°05′N, 2°34′W	2	July 2008	0 [–]	0 [–]	2 (100%) [95.1% and 94.3%]
Spain (Cáceres), colony 1 39°31′N, 7°03′W	10	May–June 2009	0 [–]	1 (10%) [84.1%]	5 (50%) [89.4%, 90.8%, 94.6%, 96.9% and 98.3%]
Spain (Cáceres), colony 2 39°41′N, 6°52′W	3	May 2009	0 [–]	0 [–]	1 (33.3%) [97.3%]
Slovakia (Devinska Nová Ves) 48°12′N, 16°58′E	1	July 2008	0 [–]	0 [–]	1 (100%) [91.6%]
Slovakia (Trávnica) 48°09′N, 18°20′E	1	July 2008	0 [–]	0 [–]	1 (100%) [89.4%]
Slovakia (Sturovo) 47°48′N, 18°43′E	1	July 2008	0 [–]	0 [–]	1 (100%) [90.0%]
Kyrzygistan (Burana) 42°44′N, 75°15′E)	2	July 2008	0 [–]	0 [–]	2 (100%) [93.5% and 88.3%]
Italy (Palermo), colony 1 37°59′N, 13°53′E	4	August 2009	0 [–]	0 [–]	4 (100%) [90.6%, 91.9%, 97.5% and 98.3%]
Italy (Palermo), colony 2 37°59′N, 13°53′E	4	August 2009	0 [–]	0 [–]	4 (100%) [89.4%, 94.6%, 95.8% and 96.7%]

Briefly, to detect microsporidia-like spores, whole regurgitated bee-eater pellets were homogenized individually in 2 ml of distilled water (PCR grade). Afterwards samples were macerated in Stomacher® 80 Biomaster (Seward) using bags with filter (BA6040/STR, Seward) for 2 min at high speed and this macerate was recovered in a tube (PCR grade). The fresh preparations from the resulting homogenates were observed by Phase Contrast Microscopy (PCM, ×400, ×1000). Purified spore suspensions were prepared from all macerates that were identified as positive or negative for microsporidia-like spores by PCM, and the suspensions were filtered (55 μm mesh) and centrifuged (6 min at 800 *g*) to obtain a pellet that was resuspended in 1 ml of distilled water (PCR grade). For DNA extraction 150 μl of every homogenate was put in a 96-well plate (Qiagen) with 1 ml cells in which glass beads (2 mm of diameter, Sigma) had been previously added. At least one water blank as negative control of extraction was added every 20 samples. Covered plates were shaken in a Qiagen system at 9500 r.p.m. during 95 s. Afterwards, 30 μl of ATL buffer (Qiagen 19076) and 20 μl of Proteinase K (Qiagen 19131) per well were added and plates were incubated overnight at 56°C. Subsequently, 200 μl of 2-propanol (Panreac), 30 μl of Mag Attract® Suspension G (Qiagen) and 200 μl of AL were added per well and DNA was extracted using BS96 DNA Tissue protocol extraction in a BioSprint (Qiagen). Finally, plates with DNA extracted were frozen at –20°C until use. *Nosema apis* or *N. ceranae* species were determined as previously described (Martín-Hernández *et al.*, 2007; Higes *et al.*, 2008a), using the 321APIS and 218MITOC primers. *Nosema bombi* determination was made using BOMBICAR primers, as described in Plischuk and colleagues (2009). All the PCRs reactions were carried out in a Mastercycler® ep gradient S (Eppendorf) in a volume 50 μl using the High Fidelity PCR Master (No. 12140314001 Roche Diagnostic), 0.4 μM of each primer, 0.2 mg ml^{-1} BSA, 0.1% Triton X-100 and 5 μl of DNA template. Each PCR was analysed in QIAxcel System (Qiagen), using a QIAxcel DNA High Resolution Kit (Qiagen, No. 929002). Negative controls were analysed in parallel to detect possible contaminations. The viability of spores was determined in each regurgitated pellet and nest material sample as described in Higes and colleagues (2009c) with a stained trypan blue method. The table shows the localities where bee-eater pellets were collected, the number of pellets analysed, the date of collection and the number of pellets (and %) where *N. bombi*, *N. apis* and *N. ceranae* were detected by PCR. Viability of spores (%) in infected pellets is shown in square brackets.

Migratory birds can be involved in dispersal of microorganisms as their biological carriers, mechanical carriers or as carriers of infected haematophagous ectoparasites (Hubálek, 2004). Higes and colleagues (2008b) demonstrated the presence of viable spores of *N. ceranae* inside the regurgitated pellets of bee-eaters at a local scale. Here we show a high percentage of occurrence (100% in all localities except for western Spain) of highly viable *N. ceranae* spores in bee-eater pellets along a large geographic range encompassing locations in the Iberian Peninsula, central Europe and central Asia. In contrast, *N. apis* was detected in a single locality and *N. bombi* was not detected at all. These results confirm the widespread geographical distribution of *N. ceranae* infecting hymenoptera and its high prevalence, and agree with other reports (e.g. COLOSS Workshop, 2009; Higes *et al.*, 2010a) stressing the novel status of this aetiological agent producing an emerging disease, named nosemosis type C in infected *A. mellifera* honeybees (Higes *et al.*, 2010a). Recently Forsgren and Fries (2010) suggested that *N. ceranae* seems to have no competitive advantage over *N. apis* in mixed infections when spores are administered at the same time in a laboratory experiment. In contrast, our field data reveal a much higher prevalence of *N. ceranae* than of *N. apis*, which suggests that the former outcompetes *N. apis* under natural conditions. The extent and efficiency of the geographic dispersal of pathogens by birds depends on a variety of biotic and abiotic factors, like dispersal distance and biological cycle of the bird, spore excretion or viability and resistance of spores (Hubálek, 2004; Brown *et al.*, 2007; Lebarbenchon *et al.*, 2009). Several aspects of the system here studied suggest that bee-eaters may have contributed considerably to the dispersal of *N. ceranae*: (i) since the hunting range of bee-eaters during the breeding season does not usually exceed 5 km (range: 1–7 km, Cramp, 1985), dissemination of spores at this time is likely to occur at a short scale; however, dissemination can occur at a much

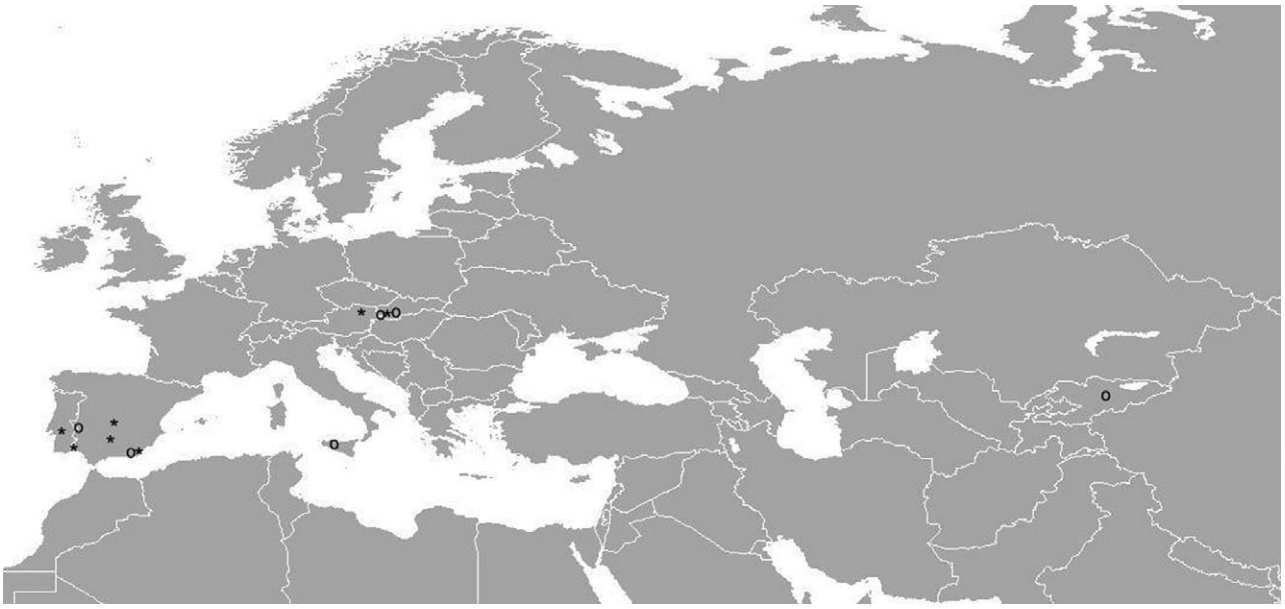

Fig. 1. Bee-eater pellets were collected during the 2008 and 2009 breeding seasons around colonies in various locations (open circles in the figure, see also Table 1). Pellets were aseptically introduced into sterile plastic tubes and kept at ambient temperature. They were submitted to the CAR laboratory and immediately analysed. Nest material from the breeding chamber of nests used the previous breeding season was collected during various years and from different locations (asterisks in the figure, see also Table 2). Samples were collected in plastic bags and stored at room temperature until their analysis.

larger scale during the long spring and winter migration journeys, when birds can feed on infected honeybee foragers since apiaries are usual stopover sites during migration (Yosef *et al*., 2006), (ii) pellet/spore excretion, viability and resistance of spores. If the frequency of spore excretion is short and the inactivation rate of spores in natural conditions is high, dissemination over long distances is unlikely to occur unless birds migrate at high speed. For the bee-eaters, the frequency of pellet/spore excretion is likely short (every 1.30–4 h after foraging for captive bee-eaters, Cramp, 1985). Concerning viability of spores, Higes and colleagues (2008b) reported a high viability for at least 18 days and Fenoy and colleagues (2009) showed that spores were highly resistant to high temperature and desiccation but not to freezing. Since bee-eaters can migrate at high speed (more than 500 km in a day, Cramp, 1985), it is very likely that viable spores can be spread at long distances during the spring and autumn migration. This could explain the occurrence of *N. ceranae* in far-out, isolated locations. For example, this microsporidium has been detected in honeybee colonies in the island of Ouessant (France) (Colin *et al*., 2008; 2009). This isolated location has been free for other bee pathogens (*Varroa destructor* and correlated viruses) (Tentcheva *et al*., 2004), probably due to the strict isolated regime since 1976 (Colin *et al*., 2008; 2009), because these pathogens need biological material as adult honeybees and bee brood for their widespread dispersal. But this scenario is not valid for *N. ceranae* because bee-eaters are common migrant visitors (F. Quénot, pers. comm.) and probably an important and recent source for infective spores in this and other islands.

Bee-eaters could eventually have some beneficial effects for honeybee colonies. Since birds usually prey on forager bees, which are the population containing the highest spore burdens (Higes *et al*., 2008a; Meana *et al*., 2010), and since the presence of *Nosema* spp. may influence the flight behaviour of forager bees (Kralj and Fuchs, 2009), avian predators could selectively prey on infected foragers and reduce their proportion in the colonies. By carrying infected insects to the nests, bee-eaters could also sequester spores in their burrows. We detected the presence of microsporida-like spores in nest material from most locations by PCM methods. However PCR techniques gave negative results. Several reasons can explain this apparent disagreement: (i) the integrity (and thus detectability by genetic procedures) of the spores in the material collected is probably low (note that viability of spores in nest material is nil in all cases, Table 2) due to the time elapsed from collection (2002–2007) until the analyses were performed (2009). The fact that the only samples where spores were not found by PCM were the oldest ones supports this view; (ii) the amount of spores in the subsamples under analysis could be low due to deterioration by the microclimate in the nests, removal by detritivorous insects that feed on the organic matter accumulated in the nests and the subsampling required for the analysis of this earthy material (see legend of Table 2).

Table 2. Spore detection in nest material was performed in subsamples of 5–10 g that were taken after thoroughly mingling the original samples.

Location	No. of samples	Date	Samples with positive detection by PCM (%) [Viability]
Spain (Almería) 37°09′N, 2°13′W	2	2002	0 [–]
Spain (Córdoba), colony 1 37°48′N, 5°66′W	3	2002	0 [–]
Spain (Córdoba), colony 2 37°38′N, 4°41′W	3	2005	100 [0.0]
Spain (Córdoba), colony 3 38°18′N, 4°37′W	1	2005	100 [0.0]
Spain (Huelva), colony 1 37°35′N, 6°45′W	6	2005	100 [0.0]
Spain (Huelva), colony 2 37°56′N, 6°41′W	2	2006	100 [0.0]
Spain (Madrid) 40°32′N, 3°27′W	2	2002	0 [–]
Portugal (Castro Verde) 37°41′N, 8°04′W	3	2005	66.6 [0.0]
Austria (Wagram) 48°14′N, 14°11′E	5	2005	40.0 [0.0]
Slovakia (Gbelce) 47°51′N, 18°32′E	3	2007	100 [0.0]

After manually removing rough material (e.g. gravel, stones), subsamples were individually suspended in 50 ml of distilled water (PCR grade) and filtered (55 μm mesh). The result was centrifuged (6 min at 800 *g*) to obtain a pellet that was resuspended in 1 ml of distilled water (PCR grade). From then onwards nest material samples were analysed with the same procedure followed for bee-eater pellets. The table shows the localities, the number of samples analysed and year of collection of bee-eaters nest material. No spore was detected by PCR methods but 63% of the samples were positive to *Nosema* spores by PCM methods. Viability of spores (%) in infected pellets is shown in square brackets.

More research on vulnerability of infected bees and selective predation by bee-eaters and on the role of nests as sinks for *Nosema* spores is necessary before supporting a beneficial role of bee-eaters on infected colonies.

Our study also provides additional pieces of information on the epizootiological status of nosemosis: (i) the high prevalence of *N. ceranae* suggests that the microsporidium is widespread under natural conditions in forager bees (see Higes *et al.*, 2010a), which are usually preyed upon by bee-eaters, (ii) the prevalence of *N. ceranae* is much higher than the one of *N. apis*, considered till recently the most common microsporidium infecting honeybees, and (iii) the prevalence of *N. bombi* is nil in agreement with previous information reporting the low prevalence of this microsporidium in Hymenoptera (Plischuk *et al.*, 2009; Sokolova *et al.*, 2010). These results agree with data collected in other geographical areas (Martín-Hernández *et al.*, 2007; Topolska and Kasprzak, 2007; Higes *et al.*, 2008a; 2009a; Plischuk *et al.*, 2008; 2009; Giersch *et al.*, 2009; Tapaszti *et al.*, 2009) and, following the criteria by Brooks (1979) for insect microsporidia, support the epizootic conditions of nosemosis due to *N. ceranae* in Europe and the enzootic status of nosemosis due to *N. apis*.

Since the dispersal ability of many microorganisms is directly linked to bird dispersal, avian mobility and migration are potentially crucial epizootiological factors (e.g. Hubálek, 2004) that should not be disregarded when drawing conclusions about the transmission of pathogens. Conclusive evidence of the spread of *N. ceranae* by bee-eaters would require tracking the movements of marked birds after feeding on infected bees and analysing the pellets expelled during their flights. Given the unfeasibility of this approach, this article shows that the European bee-eater has a potential to spread the pathogen *N. ceranae*, aetiological agent of a new emergent nosemosis, at a large, continental scale. The pellets analysed in this study were collected mainly at the end of the breeding season, when bee-eaters aggregate to start migration. Therefore, spread of *N. ceranae* by bee-eaters is, in our opinion, very likely. This is even more the case if we consider the likely dispersal of viable spores by avian faeces (see, for instance, Snowden *et al.*, 2001). Even though Higes and colleagues (2008b) found no viable spores in bee-eater droppings, the low number of samples analysed does not exclude this possibility.

The European bee-eater is just one of the 26 species of bee-eaters in the world. Most of these species are found in Africa but some of them also occur in Asia and Australia (Fry, 2001). Thus, it is very likely that the dissemination of *Nosema* spores by bee-eaters is occurring at a continental scale by means of different bird species. Whereas bee-eaters do not occur in the New World, other bird species feeding on bees (e.g. Kingbirds, Mockingbirds, Tyrant flycatchers) could have contributed to the dispersal of the pathogen. Factors affecting the movements of these birds (like changes in land use and agricultural practices, climate change, Kinzelbach *et al.*, 1997) could influence the spread of the disease.

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