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SHORT COMMUNICATION

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Survival of honey bees *(Apis mellifera)* infected with *Crithidia mellificae* spheroid forms (Langridge and McGhee: ATCC® 30254™) in the presence of *Nosema ceranae*

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Abstract

Crithidia mellificae, a trypanosomatid parasite of *Apis mellifera,* has been proposed to be one of the pathogens responsible for the serious honey bee colony losses produced worldwide in the last decade, either alone or in association with *Nosema ceranae.* Since this pathogenic effect contradicts the results of the experimental infections originally performed by Langridge and McGhee nearly 40 years ago, we investigated the potential linkage of this protozoan with colony decline under laboratory conditions. *Nosema*-free and trypanosomatid-free honey bees from three different colonies were experimentally infected with fresh *C. mellificae* spheroid forms (reference strain ATCC30254), with *N. ceranae* fresh spores and with both parasites at the same time. Replicate cages were kept at 27 °C and used to analyse survival. *C. mellificae* spheroid forms did not reduce significantly the survival of the worker bees (64.5% at 30 days post-infection *vs*. 77.8% for the uninfected bees used as controls; differences were non statistically significant) under these experimental conditions. In contrast, the cages infected with *N. ceranae* exhibited higher rates of mortality from the 20th day post-infection onwards, irrespective of the presence of *C. mellificae,* suggesting that the spheroid forms of the latter have no pathological effect on *A. mellifera.*

Additional key words: trypanosomatids; microsporidia, colony collapse.

Abbreviations used: CCD (colony collapse disorder), dpi (days post-infection), PCR (polymerase chain reaction).

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Introduction

In the last decade, the phenomenon of honey bee colony death has attracted the interest of scientists, beekeepers and the general public alike. Increased annual honey bee colony losses have been associated with many factors, including viruses, bacteria, fungi, mites and trypanosomatids (Higes *et al.*, 2006; vanEngelsdorp *et al.*, 2009; Cox-Foster *et al.*, 2007; Dainat *et al.*, 2012; Francis *et al.*, 2013; Ravoet *et al.*, 2013; Cepero *et al.*, 2014). Trypanosomatids are flagellated protozoan parasites and they are the direct descendants of the first eukaryotes that appeared on the earth ~1.5 billion years ago (Knoll, 1992). Several genera

of the Trypanosomatidae cause disease in humans, domestic animals, plants (Galanti *et al.*, 1998) and insects (Schmid-Hempel & Tognazzo, 2010). Indeed, the interest in insect trypanosomatids, especially those affecting bees, has increased among researchers due to their possible involvement in colony collapse disorder (CCD). Until now honey bees were thought to be infected by only a single trypanosomatid species, *Crithidia mellificae*, the trypanosomatid first detected in Australia during the diagnostic examination of *Apis mellifera* apiaries (Langridge & McGhee, 1967). The original experimental infection with *C. mellificae* suggested it had no pathological effects on this hymenopteran host, which explains why this organism has been largely ignored for more than 40 years.

However, a relationship between *C. mellificae* infection and colony losses was recently proposed in the USA (Runckel *et al.*, 2011) and Belgium (Ravoet *et al.*, 2013). Indeed, *C. mellificae* was 6.15-fold more abundant in CCD-affected colonies than in unaffected colonies in a colony-cohort study, and its detection was strongly associated with the presence of *N. ceranae* (Runckel *et al.*, 2011). In addition, a high prevalence of *C. mellificae* (70.5%) was reported in Belgium (Ravoet *et al.*, 2013), and a correlation was detected between *C. mellificae* incidence in July and winter mortality. This study also confirmed the link between *C. mellificae* and *N. ceranae* previously described in the USA (Runckel *et al.*, 2011), suggesting that co-infection by these pathogens has a negative synergistic impact on colony longevity (Ravoet *et al.*, 2013). More recently, a holistic screening of professional apiaries with typical honey bee depopulation symptoms in Spain (Cepero *et al.*, 2014) revealed a higher prevalence of *N. ceranae* with only a moderate presence of trypanosomatids. Genetic analyses of the 18S rDNA and GAPDH sequences of these trypanosomatids proved that they were phylogenetically distant from those of the reference ATCC30254 *C. mellificae* strain, providing the first hard evidence of honey bee infection by a different lineage (Cepero *et al.*, 2014). The existence of this taxon, which was later named *Lotmaria passim* (Schwarz *et al.,* 2015), had been previously suggested in the USA (Cornman *et al.*, 2012). It is also worth noting that all the sequences attributed to *C. mellificae* until then, including its draft genome assembly (Runckel *et al.*, 2014), were more closely related to this new taxon than to the "true" *C. mellificae* (Cepero *et al.*, 2014).

Together, these studies inspired renewed interest in this pathogen and its relationship with the highly prevalent microsporidium *N. ceranae* as one of the possible causes of bee colony collapse in different parts of the world. Given the discrepancies regarding the pathological effect of *C. mellificae* in honey bees (Langridge & McGhee, 1967; Ravoet *et al.*, 2013), we evaluated the effect of experimentally infecting bee colonies under laboratory conditions with the *C. mellificae* ATCC30254 reference strain, with or without *N. ceranae*. In this way we were able to specifically assess the influence of *C. mellificae* on the bees' life expectancy and determine whether a synergistic effect of this pathogen with *N. ceranae* could be detected.

Material and methods

To perform these experiments, the *C. mellificae* reference strain ATCC30254 was first cultured in ATCC medium 355 as recommended, and it was further sub-cultured in solid medium as described by Popp & Lattorff (2011). Visible, isolated colonies were selected and resuspended in milliQ water (PCR-quality) to obtain trypanosomatid spheroid forms for experimental infection (Schwarz & Evans, 2013). The DNA from those colonies was extracted as described previously in Cepero *et al.* (2014) and the trypanosomatid species was confirmed by PCR (Meeus *et al.*, 2010) and subsequent sequencing (Cepero *et al.*, 2014). Freshly purified *N. ceranae* spores for experimental infection were obtained as described elsewhere (Martín-Hernández *et al.,* 2011, 2012; Higes *et al.,* 2013), counting both the *N. ceranae* spores and *C. mellificae* spheroid forms in a haemocytometer. DNA was extracted from an aliquot of *Nosema* spores to confirm the species by PCR (Martín-Hernández *et al.*, 2012). *Nosema*-free and trypanosomatid-free honey bees were obtained as described previously (Higes *et al.*, 2013). Emerging worker bees from three different colonies were removed carefully, confined to cages in groups of 25 and kept in the incubator for 5 days at 27ºC, the optimum temperature for *C. mellificae* growth (Langridge & McGhee, 1967). Sixteen replicate cages were prepared, 12 of which were used to analyse survival: three cages infected with fresh *N. ceranae* spores; three with fresh *C. mellificae* spheroid forms; three co-infected with *N. ceranae* and *C. mellificae*; and three that remained uninfected (used as controls).

Honey bees (*Apis mellifera iberiensis*) were infected as described elsewhere (Higes *et al.*, 2013), administering 1 µL of a sucrose solution containing 50,000 *N. ceranae* spores or 50,000 *C. mellificae* spheroid forms. The co-infected bees received 1 µL of each pathogen solution, while uninfected control bees were fed with 2 µL of the 50% sucrose solution alone. After inducing infection, the bees in each cage

were fed ad libitum with 50% sugar syrup + 2% Promotor L (Calier Lab). Infected and uninfected bees were kept at 27 °C in separate incubators (Memmert ® Mod. IPP500, 0.1 °C), and the replicate cages were observed daily. Cumulative honey bee mortality was analysed using a Kaplan-Meier Log-Rank survival analysis and the slope of the survival function was analysed according to the Mantel-Cox test with the aid of IBM Statistics V22.0.

Results and discussion

The mortality of the workers infected with *N. ceranae and N. ceranae+C. mellificae* was significantly higher than that of those that received the *C. mellificae* treatment alone and that of the control worker bees. Bee survival at 30 days post-infection (dpi) was 77.8%, 64.5%, 0% and 0% for the uninfected control honey bees, and the honey bees from cages infected with *C. mellificae*, *N. ceranae* and a mixture of both parasites, respectively (Fig. 1). The mortality of the workers infected with *N. ceranae* and *N. ceranae+C. mellificae* was significantly higher than that of those that received *C. mellificae* alone and that of the control worker bees (Kaplan-Meier Long-Rank test, $p<0.001$). Conversely, the survival of the control and the *C. mellificae* infected bees was significantly higher than that of the other two groups (Kaplan-Meier Long-Rank test, $p<0.001$). The slopes of the survival functions were statistically different according to the Mantel-Cox test ($p<0.05$). The evolution of control worker bee survival was similar and not significantly different from that of those infected with *C. mellificae* alone ($p>0.05$). Likewise, the *N. ceranae* and mixed infection groups also displayed a parallel evolution in terms of survival ($p>0.05$), which differed considerably from the other two groups from 20 dpi onwards. The highest mortality rates of worker honey bees occurred in the cages infected with *N. ceranae*, irrespective of the presence of *C. mellificae*.

Since the first report of *N. ceranae* infecting of *A. mellifera* bees there has been some controversy about the effects of these microsporidia. However, most of the studies published in recent years have confirmed that *N. ceranae* has a pathogenic effect on this host, expressed as a shortening of the honey bee (workers) lifespan (Higes *et al.,* 2013). Significantly, the longevity of infected bees varies strongly among these studies, probably due to the many factors that influence the development and the course of the infection. For example, this is the case of the temperature, which must be taken into consideration in laboratory studies since *N. ceranae* has been proposed to be eurithermal (Higes *et al.,* 2010). In this study, the survival of honey bees following experimental infec-

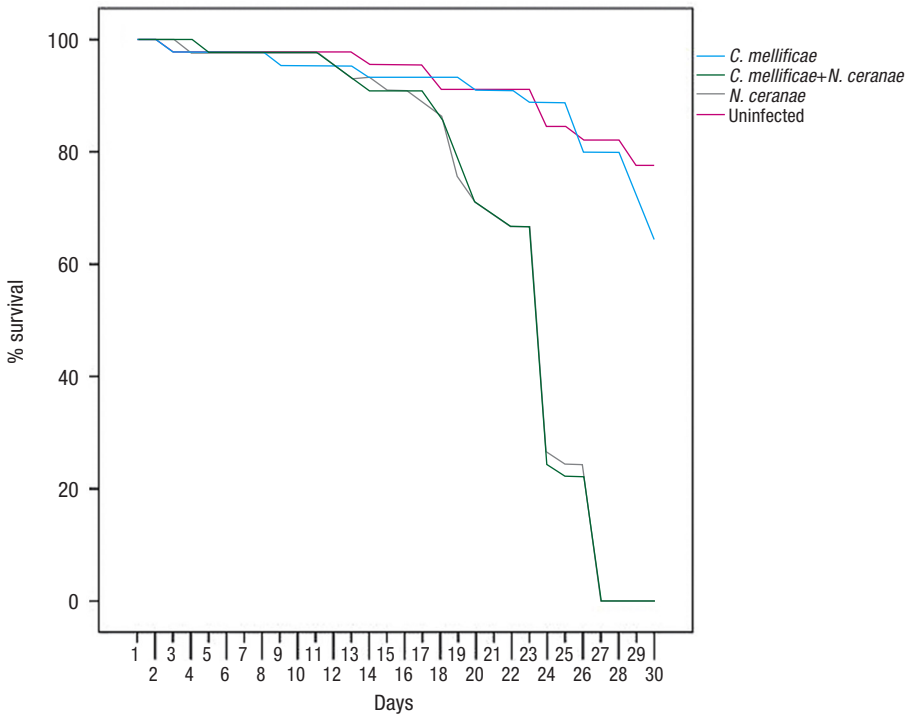

Figure 1. Cumulative honey bee mortality after infection with *Crithidia mellificae*, *Nosema ceranae and N. ceranae+C. mellificae* or uninfected (Control).

tion with *N. ceranae* is consistent with that seen previously (reviewed by Higes *et al.*, 2013; Williams *et al.*, 2014; Huang *et al.*, 2015; Milbrath *et al.*, 2015), confirming that bees infected with *N. ceranae* have a shorter life expectancy than uninfected bees. The incubation temperature used may explain the distinct evolution in the survival of infected honey bees relative to other laboratory experiments performed in our laboratory (Martín-Hernandez *et al.*, 2009).

Previous co-infection experiments with *N. ceranae* and *C. mellificae* under laboratory conditions have shown to modify the bees's immune response in a distinct manner to that produced when bees were exposed only to one pathogen or another. Mixed *C. mellificae* and *N. ceranae* infection altered the repertoire of systemic antimicrobial peptides and reduced cellular immunity at the level of the immune response, although the mortality of the infected bees was not recorded (Schwarz & Evans, 2013). An epidemiological link between the presence of *C. mellificae* and bee colony collapse has been suggested in field conditions, either alone or in association with *N. ceranae* (Runckel *et al.*, 2011; Ravoet *et al.*, 2013), although no information regarding worker bee mortality was reported. The effect of the infection with *C. mellificae* spheroid forms on honey bee survival suggests that such cellular stage does not alter the insect's longevity, and does not significantly modify the mortality of bees co-infected with *N. ceranae* when compared to that of the bees infected with the microsporidium alone. These results are consistent with those of Langridge & McGhee (1967) suggesting that *C. mellificae* has no pathological effect on honey bees. However, further research is needed to determine the development of this trypanosomatid in different culture media, investigate its life cycle and the possible variability of its pathogenic capacity. The finding of a new trypanosomatid taxon in collapsed colonies under field conditions raises new questions about the role of *C. mellificae* in honey bee losses, an issue that should be addressed through appropriate experimental infection and epidemiological analyses.

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