Original Article

LOTMARIA PASSIM AS THIRD PARASITE GASTROINTESTINAL TRACT OF HONEY BEES LIVING IN TREE TRUNK

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Abstract

Honey bees (*Apis mellifera* L.) inhabiting trees in forests are not managed by humans or treated for pathogens; therefore, many researchers and beekeepers believe that viral, bacterial, and parasitic diseases may lead to their decline. The aim of the study was to evaluate the prevalence of *L. passim* and *Nosema* spp. in feral colonies by real-time PCR. This study was performed on twenty-six samples of honey bees inhabiting tree trunks in north-eastern Poland. One sample consisted of sixty worker bee abdomens collected from hollow trees. Honey bees were sampled only from naturally colonized sites. Amplicons of the three evaluated pathogens were detected in twenty of the twenty-six tested samples. A significant correlation was observed between infection with three pathogens (*N. apis, N. ceranae, L. passim*) (r = 0.84) compared to infection with only two pathogens (*N. apis* and *N. ceranae*) (r = 0.49). *N. ceranae* was the predominant pathogen, but infections with various severity caused by *L. passim* were also noted in fourteen of the twenty-six tested samples. In view of the general scarcity of epidemiological data concerning coinfections with *Nosema* spp. and *L. passim* in honey bees in tree trunks in other countries, further research is needed to confirm the effect of concurrent pathogenic infections on the decline of bee colonies.

Keywords: co-parasitism, *Lotmaria passim, Nosema apis, Nosema ceranae,* real-time PCR, tree trunks

INTRODUCTION

Honey bees (*Apis mellifera* L.) initially colonized deciduous forests and trees that offered shelter as well as pollen and nectar for sustenance (Ruttner, 1988; Crane, 1999), but their habitats gradually moved from forests to human settlements (Crane, 1999; Banaszak, 2009). Bee habitats were also transformed by forest clearing (De la Rua et al., 2009, Potts et al., 2010). Nevertheless, feral honey bees still colonize tree trunks and log hives world, including Poland (Seeley, 2007; Oleksa, Gawroński, & Tofilski, 2013; Ilyasov et al., 2015) or are specially introduced to preserve biodiversity. These bees are not managed by humans or treated for pathogens, and therefore many researchers and beekeepers believe that viral, bacterial, and parasitic diseases may lead to their decline (Sammataro, Gerson, & Needham, 2000; Moritz et al., 2010; Neumann & Carreck, 2010; Rosenkranz, Aumeier, & Ziegelmann, 2010; Martin et al., 2012; van Dooremalen et al., 2012). However, not only feral honey bees are threatened with extinction, and despite high beekeeping standards and veterinary care, apiaries around the world have also been experiencing a collapse of bee colonies. The agricultural sector has sustained massive losses due to the decline of feral bees and honeybees, which play an important role in the preservation of biological diversity and sustainable farming (De la Rua et al., 2009; Moritz et al., 2010). Bees are among the most effective pollinators of entomophilous plants around the world, and the high quality of crops pollinated by bees generates environmental and economic benefits. For these reasons,

considerable research has been devoted to bee health and factors that influence the vitality and performance of honey bee colonies (Neumann & Carreck, 2010; Potts et al., 2010; Goulson et al., 2015; Ptaszyńska et al., 2018). *Varroa destructor* is regarded to be the most harmful parasite limiting bee colonies and capable of transmitting bee viruses (Rosenkranz, Aumeier, & Ziegelmann, 2010; Borsuk et al., 2012; Stanimirovic et al., 2017). Other bee pathogens are also increasingly being identified, including viruses (DWV, ABPV, CBPV), bacteria (*Paenibacillus larvae*, *Melissococcus pluton*), pathogenic fungi (*Nosema apis* and *Nosema cerane*), and protozoa of the family *Trypanosomatidae* (Sokól et al., 2016; Nazzi & Le Conte, 2016; Stevanovic et al., 2016; Łopieńska-Biernat et al, 2017; Michalczyk et al., 2018).

Trypanosomatidae are unicellular flagellates and obligatory parasites infecting various host mammals, insects and plants (Barret et al., 2003). These protozoa are generally found in the host's intestines (Maslov et al., 2013). They have been detected in insects of the order Hymenoptera and in bumblebees (Schlunsch et al., 2010). Honeybees are infected mostly by the *Crithidia mellificae* and *Lotmaria passim* species of the family *Trypanosomatidae*. (Langridge & McGhee, 1967; van Engelsdrop et al., 2009; Cornman et al., 2012; Ravoet et al., 2013; Schwarz, 2014; Arismendi et al., 2016). At present, *L. passim* is the predominant pathogenic protozoan species in the world (Schwarz et al., 2015). These protozoa have been widely investigated due to their detrimental effect on bees' behavior, physiology and immune function (Gegear et al., 2005; Runckel et al., 2011; Ravoet et al., 2013; Evans & Schwarz, 2013). Recently, increasing attention has been paid on infections caused by *Trypanosomatidae* and microsporidian parasites of the genus *Nosema* (Stevanovic et al., 2016; Hubert et al., 2017; Tritschler et al., 2017).

N. apis and *N. ceranae* microsporidia are highly specialized unicellular eukaryotic parasites (Adl et al., 2005). These pathogens are responsible for nosemosis, one of the most widespread bee diseases in the world. Bees are infected by spores ingested with water or pollen (Webster et al., 2004; Chen et al., 2008). The spread of nosemosis is largely influenced by weather conditions in different seasons of the year. Sulborska et al. (2019) have reported that *Nosema* spp. are transmitted with the wind. *N. ceranae* is regarded as the most ubiquitous endoparasite of honey bees, but *N. apis* is also frequently encountered in Poland and in the world (Fries, 2010; Higes et al., 2013). However, the infection dynamics and the interactions between *Nosema* spp. and *L. passim* in honey bees inhabiting tree trunks and log hives have never been investigated. This study is the first attempt in both Poland and the world to determine the severity of infections caused by *Nosema* spp. and *L. passim* in tree trunks. In this study we aimed to evaluate the prevalence of *Nosema* spp. and *L. passim* in bee inhabiting tree trunks by real-time PCR and determine relationships in the occurrence of these pathogens.

MATERIAL AND METHODS

Bee samples

The study was performed on twenty-six samples of bees (colonies) collected from tree trunks in north-eastern Poland (21°04'0"-21°41'0"E and 53°09'0"-53°29'0"N). Feral honey bees were sampled only from naturally colonized sites. No clinical disease symptoms like diarrhea, inability to fly, paralysis, gathering in small groups, dead bees on the bottom of the hive with swollen abdomen and legs retracted below the chest, or reduced activity were observed in these colonies. The experiment involved colonies with a life span of at least three seasons, during which the insects were monitored. Workers were sampled from every colony with the use of an open 0.9 l jar that was placed under the exit hole in a hollow tree. When bees entered the jar, the jar was capped, transported to the laboratory and stored at a temperature of -20°C.

DNA analysis

The collected twenty-six samples were analyzed with the use of molecular methods to identify and quantify *Nosema* spp. and *L. passim*. Each test was performed on a random sample of sixty worker bee abdomens collected from hollow trees. Whole abdomens of sixty bees per

sample were homogenized with a homogenizer. The whole homogenate from each sample was taken for DNA extraction. Genomic DNA was isolated with the Genomic MiniKit (A&A Biotechnology, Gdynia, Polska) according to the manufacturer's instructions. Real-time PCR was conducted with the use of FastStart Essentials DNA Green Master (Roche). The 20-µl reaction mixture contained ca. 150 ng of isolated DNA (1 µl), 10 µl of FastStart Essential DNA Green Master, and 0.5 µl of each primer (with a final concentration of 0.5 µM). It was supplemented to 20 µl with FastStart Essential DNA Green Master Water (PCR grade). The qPCR reaction was carried out in a Light Cycler Nano System thermal cycler (Roche). The reactions were run using a thermal profile consisting of a hold at 95°C for 10 min, followed by 40 cycles of a 2-step amplification consisting of 95°C for 10 s, primer annealing at 54°C for 60 s and 72°C for 15 s, melting at 65°C for 60 s and 95°C for 1 s, and a final hold at 45°C for 600 s. *Lotmaria passim* was identified with primers LpCytb_F2: 5'-AGTaTGAGCaGTaGGtTTTaTTATa-3' and LpCytb_R: 5'-gcCAaAcACCaATaACtGGtACt-3 (Vejnovic et al., 2018).

Nosema apis and *N. ceranae* were identified and quantified with FastStart Essentials DNA Green Master (Roche). The 20-µl reaction mixture contained approximately 150 ng of isolated DNA (1 µl), 10 µl of FastStart Essential DNA Green Master, and 0.5 µl of each primer. It was supplemented to 20 µl with FastStart Essential DNA Green Master Water (PCR grade). The following primers were applied: 321APIS FOR (5'-GGGGGCATGTCTTTGACGTACTATGTA-3') and 321APIS REV (5'-GGGGGGCGTTTAAAATGTGAAACAACTATG-3') for *N. apis*, and 218 MITOC REV (5'-CCCGGTCATTCTCAAACAAAAAACCG-3') for *N. ceranae* (Martı'n-Hernandez et al. 2007). The reactions were run using a thermal profile consisting of a hold at 95°C for 600 s, followed by forty-five cycles of a three-step amplification consisting of 95°C for 20 s, 55°C for 20 s, and 72°C for 15 s, melting at 65°C for 60 s and 95°C for 1 s, and a final hold at 45°C for 600 s.

In the real-time PCR assay, *L. passim, N. apis* and *N. ceranae* parasites were quantified in bees inhabiting tree trunks with the use of DNA standards (University of Belgrade, Faculty of Veterinary Medicine, Department of Biology, Serbia). In each reaction, standard curves were generated by ten-fold serial dilutions of known concentrations of *L. passim*, *N. apis*, and *N. ceranae* DNA for each parasite separately. Each run contained negative and positive control and experimental samples preformed in duplicates. The gene copy number of *L. passim* and *Nosema* spp. were calculated using software program LightCycler Nano (Roche).

Statistical analysis

The statistical analysis was performed using SAS software Version 9.5 (Statistical Analysis System Institute, Cary, NC). The correlation between the analyzed pathogens was determined through the calculation of Spearman's rank correlation coefficient *r*. The number of copies of the genome *Nosema* spp. and *L. passim* identified on the bees were compared using the one-way ANOVA, followed by Tukey test. Differences at $p \leq 0.05$ were considered significant.

RESULTS

After the examination of twenty-six feral honeybee colonies, we found that *N. ceranae* was the predominant pathogen. The presence of *N. ceranae* was identified in twenty-five samples (96.15%). *N. apis* in twenty samples (76.92%), *L. passim* in fourteen samples (53.84%); both pathogens were always accompanied by *N. ceranae*. While *Nosema ceranae* alone was detected in only two cases. Coinfections with *N. ceranae/L. passim* were only noted in three samples (11.5%), coinfections with *N. apis/N. Ceranae* in nine samples (34.61%) and multiple infections with *N. ceranae/N. apis/L. passim* in eleven samples (42.3%) (Fig.1). Statistically significant ($F=25.17$ $p=0.000001$) variations in the average gene copy number of *L. passim* and *Nosema* spp. were observed in the bees (Fig. 2). A significant correlation was observed between infection with the three analyzed pathogens (*N. apis, N. ceranae,*

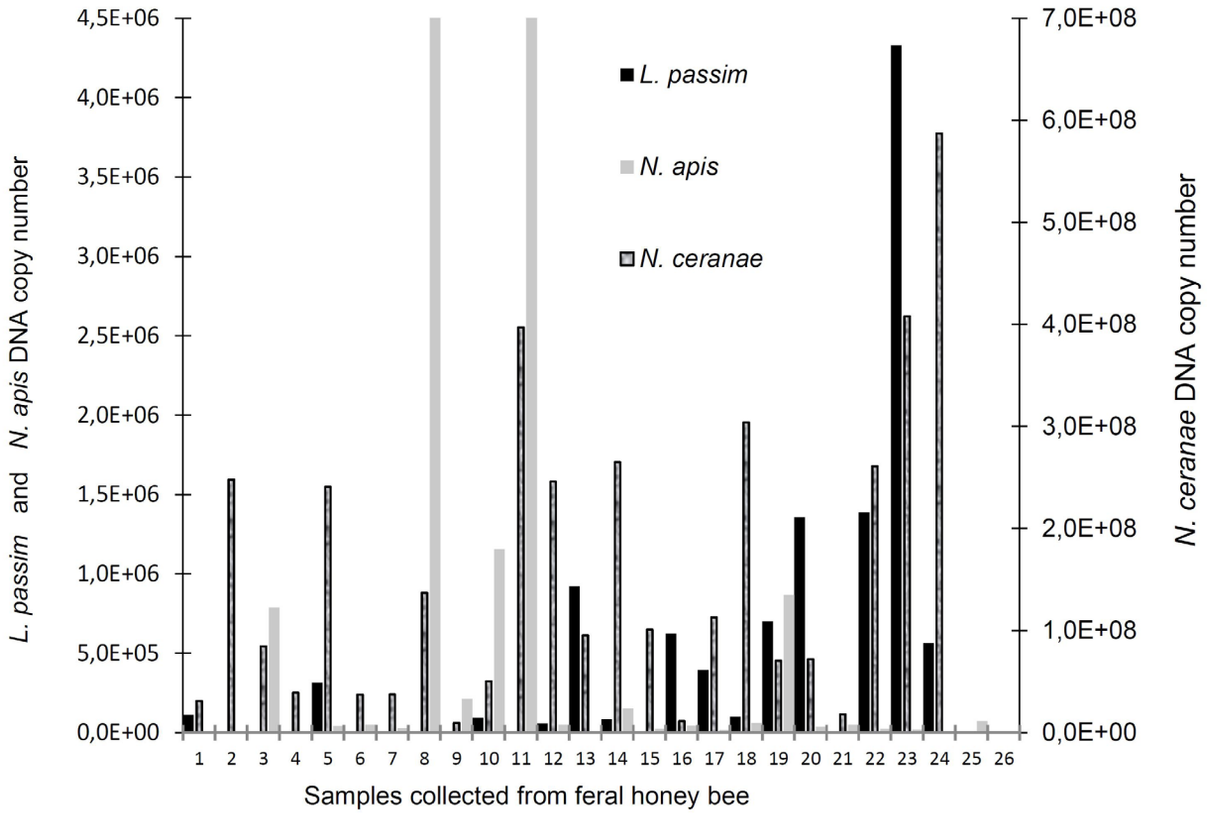

Fig. 1. Severity of infections caused by *L. passim* and *Nosema* spp. in bee samples.

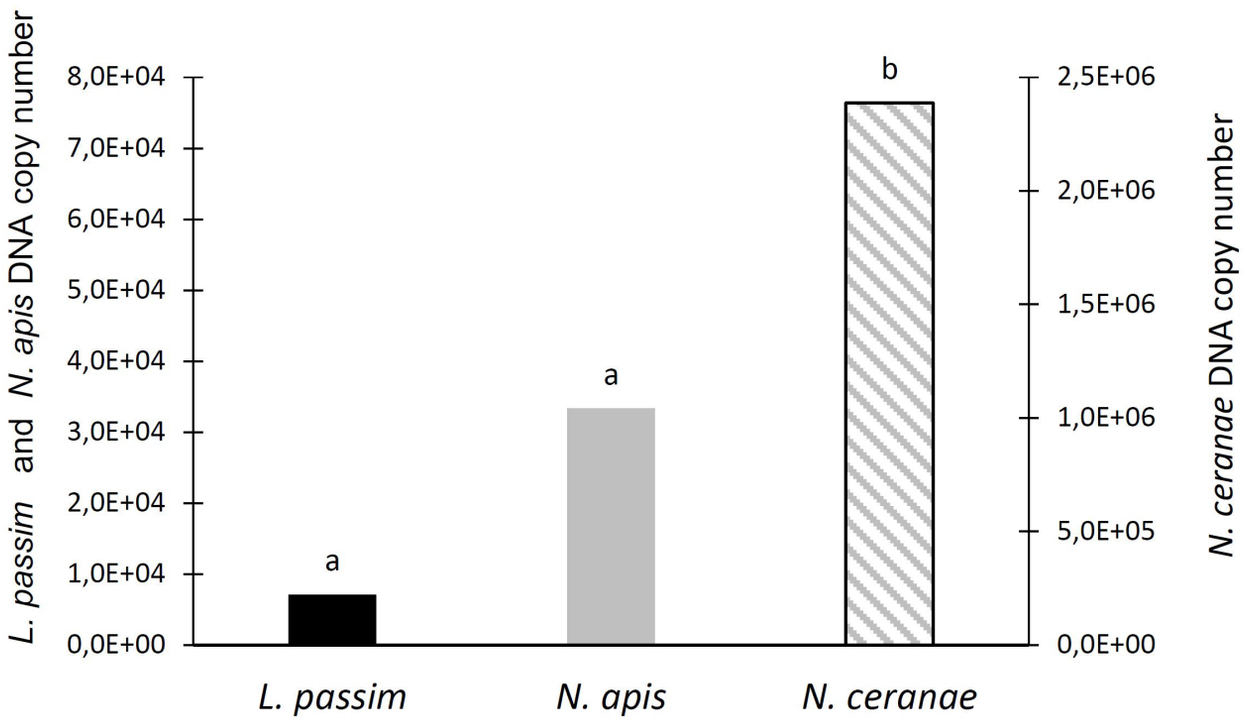

Fig. 2. Average number of copies of the genome per bee.
a, b – the different small letters denote statistically significant differences between the number of copies of the genome of *Lotmaria passim* and *Nosema* spp. (one-way ANOVA; Tukey test, $p \leq 0.05$)

L. passim) (r = 0.84) compared to infection with two pathogens only (*N. apis* and *N. ceranae*) (r = 0.49).

DISCUSSION

Crithidia mellificae (Langridge & McGhee, 1967) were initially regarded as the most prevalent pathogen of family *Trypanosomatidae* in honeybee colonies (Ravoet et al., 2013). However, Schwarz et al. (2015) demonstrated that honeybees were most frequently infected by *L. passim*. Many isolates that had earlier been identified as *C. mellificae* were reclassified as *L. passim*, and the latter species is presently regarded as the predominant protozoan species in bees worldwide (Schwarz et al., 2015). In this study, we attempted to evaluate the prevalence of *L. passim* and *Nosema* spp. in feral honeybees and to determine potential relationships between the occurrence of these parasites.

As many researchers have demonstrated, *L. passim* can compromise the health of honeybees, and coinfections with other pathogens can exacerbate colony losses. Tritschler et al. (2017) reported a relationship between the occurrence of *Nosema* spp. and *L. passim* in honeybees. Contrary results were reported by Vejnovic et al. (2018), who attributed the observed variations in the prevalence of *Nosema* spp. and *L. passim* to different methodological approaches. Vejnovic et al. (2018) studied pooled samples, whereas Tritschler et al. (2017) examined individual bees. Research results are also affected by weather conditions and differences in the prevalence of *Nosema* spp. in various countries (Sulborska et al., 2019). These pathogens have been identified in 46.7% of bee colonies in Switzerland (Retschnig et al., 2017) and in 95.7% of bee colonies in Serbia (Stevanovic et al., 2016).

Bee colonies are also increasingly being infected with *N. ceranae* in Poland, which was confirmed in our previous study (Michalczyk, Sokół, & Koziatek, 2015). *Nosema ceranae* was also the predominant pathogen in the feral honeybee samples analyzed in the present study (Fig. 2). *Lotmaria passim* always co-occurred with *Nosema* spp., and considerable differences in the abundance of pathogens were noted between tree trunks. In contrast, Vejnovic et al. (2018) reported no correlation between the occurrence of *N. ceranae* and *L. passim*. In the current study, nearly all monitored colonies were infected with *N. ceranae*, and coinfections with *L. passim* were additionally observed in fourteen samples. Real-time PCR supported accurate quantification of *Nosema* sp. and *L. passim* in the evaluated colonies. Our data presented significantly more cases with *L. passim* infection with both *Nosema* spp. compared to cases where *L. passim* infection was accompanied by a single *Nosema* spp. This may be due to the exhaustion of the bee's organism and complete loss of its defense capabilities. Therefore, it is subjected to the penetration of the third parasite *L. passim*. A similar relationship was observed by Ptaszyńska, Paleolog, & Borsuk (2016) and Borsuk et al. (2013). The authors found that *N. ceranae* infection promoted the proliferation of yeasts in honey bee intestines. In our case, the invasion of three pathogens was intensified, perhaps due to the compromised immunity of the honeybees. *N. ceranae* (Antùnez et al., 2009) suppresses the immune system, which facilitates the penetration of other parasites into the bee's organism and additional deterioration. This in turn leads to the depopulation and extinction of bee colonies. From the etiological point of view, it will be important to establish which pathogen is the first to colonize the host in future studies. The role played by *L. passim* in honey bees colonies is still being debated, but there are no doubts that this parasite compromises bee health. A steady increase in the mortality of bee colonies and lower levels of bee activity were reported by Chilean beekeepers (Arismendi et al., 2016). These losses were probably sustained due to the presence of *N. ceranae* in 18% of the studied apiaries, but *L. passim* was also detected in 90% of the analyzed samples (Rodríguez et al., 2012; Rodríguez et al., 2014). A similar study was conducted in Belgium by Ravoet et al. (2013), who identified *C. mellificae* as a new pathogen responsible for the collapse of bee colonies.

In view of the general scarcity of epidemiological data about coinfections with *Nosema* spp. and *L. passim* in feral honey bees in other countries, further research is needed to confirm the effect of concurrent pathogenic infections on the decline of bee colonies. The role of *L. passim* and coinfections with other pathogens, including viral, bacterial and parasitic infections, in bee colonies should be investigated.

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