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# Evidence for emerging parasites and pathogens influencing outbreaks of stress-related diseases like chalkbrood

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ABSTRACT

In agriculture, honey bees play a critical role as commercial pollinators of crop monocultures which depend on insect pollination. Hence, the demise of honey bee colonies in Europe, USA, and Asia caused much concern and initiated many studies and research programmes aiming at elucidating the factors negatively affecting honey bee health and survival. Most of these studies look at individual factors related to colony losses. In contrast, we here present our data on the interaction of pathogens and parasites in honey bee colonies. We performed a longitudinal cohort study over 6 years by closely monitoring 220 honey bee colonies kept in 22 apiaries (ten randomly selected colonies per apiary). Observed winter colony losses varied between 4.8% and 22.4%; lost colonies were replaced to ensure a constant number of monitored colonies over the study period. Data on mite infestation levels, infection with viruses, *Nosema apis* and *Nosema ceranae*, and recorded outbreaks of chalkbrood were continuously collected. We now provide statistical evidence (i) that *Varroa destructor* infestation in summer is related to DWV infections in autumn, (ii) that *V. destructor* infestation in autumn is related to *N. apis* infection in the following spring, and most importantly (iii) that chalkbrood outbreaks in summer are related to *N. ceranae* infection in the preceding spring and to *V. destructor* infestation in the same season. These highly significant links between emerging parasites/pathogens and established pathogens need further experimental proof but they already illustrate the complexity of the host–pathogen-interactions in honey bee colonies.


## 1. Introduction

Managed colonies of the western honey bee *Apis mellifera* L. provide more than 90% of the commercial pollination of crop monocultures, particularly of specialty crops such as nuts, berries, fruits and vegetables, whose agricultural production is dependent on insect pollination (Allsopp et al., 2008; Klein et al., 2007). Therefore, honey bees are the most important commercial pollinator in global agriculture (Gallai et al., 2009; Morse and Calderone, 2000). The pollinator-dependent agricultural production is still increasing (Aizen et al., 2008; Aizen and Harder, 2009), therefore, the long-term declines of managed honey bee hives in the USA and some European countries (vanEngelsdorp and Meixner, 2010) became an issue of widespread interest and concern. Over the past years evidence has been accumulating that the observed decrease in honey bee vitality and increase in colony losses are largely owing to pathogens and parasites. Parasitic mites (*Varroa destructor*, *Acarapis woodi*, *Tropilaelaps* sp.), fungi (*Nosema* spp., *Ascosphaera apis*), bacteria (*Paenibacillus larvae*, *Melissococcus plutonius*), viruses, and vermins (Small hive beetle) attack honey bees worldwide (Genersch, 2010b). In addition, honey bee vitality is thought to be negatively affected by many pesticides and fungicides used in agriculture and the chronic exposure to acaricides needed to combat *V. destructor* in apiculture (Barnett et al., 2007; Desneux et al., 2007; Karise, 2007; Moncharmont et al., 2003). Both pathogenic and environmental factors acting on honey bees and honey bee colonies contribute to decreased honey bee vitality and well-being which eventually may lead to colony losses (Cox-Foster et al., 2007; Genersch, 2010b; Oldroyd, 2007; vanEngelsdorp et al., 2007). However, the interplay between these factors on both lethal and sublethal level is poorly understood.

Several of the above mentioned pests and pathogens have been identified as cause or marker of failing and collapsing colonies. In Spain, huge colony losses mainly during the season could be attributed to the honey bee pathogenic microsporidium *Nosema ceranae* (*N. ceranae*) (Higes et al., 2008, 2009, 2010a). However, in Germany *N. ceranae* could be ruled out as cause of current colony losses (Gisder et al., 2010). Instead, a long-term longitudinal study, the German bee monitoring programme, revealed that the ectoparasitic mite *V. destructor* and two viruses, deformed wing virus (DWV) and acute bee paralysis virus (ABPV), were responsible for winter losses in this region of Europe (Genersch et al., 2010). Similar results were obtained from other countries, where either certain

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virus infections or high *V. destructor* infestation levels (Cox-Foster et al., 2007; Guzmán-Novoa et al., 2010; Highfield et al., 2009; vanEngelsdorp et al., 2009) or an overall high incidence of several pathogens and parasites including viruses and *N. ceranae* (Bacandritsos et al., 2010) correlated with colony collapses.

Although the role of certain pathogens and parasites in colony losses is far from being fully explored it has at least been the focus of many surveys and studies (see special issue on colony losses in *Journal of Apicultural Research*, 2010). Due to the complex nature of interactions between several factors, such studies analysed the effect of individual factors on colony losses rather than the effect factors might have when occurring in combination. Studies of insect–pathogen interactions mostly consider the direct interaction between one disease agent and one host species. Experimental data with mixed infections on individual insects have shown synergistic or antagonistic effect between pathogens (Guzman-Franco et al., 2009; Hughes and Boomsma, 2004). Honeybee societies are long-lived and therefore very prone to become infected or parasitised by multiple organisms but limited information is available on their interaction. *V. destructor* is known to act synergistically with several viruses by acting as mechanical and/or biological virus vector (Ball, 1983; Gisder et al., 2009; Nordström et al., 1999; Shen et al., 2005a,b; Yue and Genersch, 2005). By additionally influencing the bee's immune system it may also activate covert virus infections and render the bees more susceptible to other infections (Amdam et al., 2004; Bailey et al., 1983; Gregory et al., 2005; Yang and Cox-Foster, 2007, 2005). The aim of the current study was to unravel pathogens/parasites which influence each other to further our understanding of the multifactorial process leading to weakened or collapsing colonies. The analysed data were collected as part of a monitoring project which is conducted in Germany since autumn 2004 (Genersch et al., 2010). The implication of our results in terms of future research directions in bee pathology will be discussed.

## 2. Materials and methods

### 2.1. Bee samples and field survey

A cohort of 220 colonies kept in 22 apiaries (10 randomly selected colonies per apiary) and managed by hobbyist beekeepers in the north-eastern part of Germany were monitored for viral, bacterial, and fungal infections as well as for infestation by the ectoparasitic mite *V. destructor* between autumn 2004 and spring 2010, i.e. currently for 6 years. The colonies selected for the survey were closely monitored by a professional bee inspector for the duration of the study without introducing any changes in the beekeeping practice of the beekeeper. Overwintering success and survival during the summer season were recorded individually for each colony. The winter losses we observed in our cohort during the study period varied between 22.4% (2005/2006) and 4.8% (2008/2009). To ensure a constant number of monitored colonies, lost colonies were replaced by colonies from the same apiary, preferably by nuclei made from these colonies in the previous year, but only if they were established as part of the normal beekeeping practice of the respective beekeeper.

*V. destructor* infestation levels were determined twice a year (Fig. 1) using samples of at least 100 adult bees collected in October (autumn samples) and in July (summer samples). Collected bees were individually analysed for parasitising varroa mites and the infestation level was calculated as 'number of mites per 100 bees' and given as 'percentage of infestation' (Genersch et al., 2010). Viral infections were determined using these autumn bee samples via RT-PCR-analysis for Kashmir bee virus (KBV), acute bee paralysis virus (ABPV), sacbrood virus (SBV), deformed wing virus (DWV), and Israeli acute paralysis virus (IAPV). Bacterial infections included the detection of *P. larvae* (American Foulbrood) and *M. plutonius* (European Foulbrood) using brood samples and samples of brood comb honey collected in autumn and analysed according to the methods described in the "Manual of Standards for Diagnostics and Vaccines" published by the Office International des Epizooties (OIE), the World Organization for Animal Health (Anonymous, 2008). Monitored fungal infections were *Ascosphaera apis* (*A. apis*; chalkbrood) and *Nosema* spp. (nosemosis type A or C (Higes et al., 2010b)). For *A. apis* infections only clinical outbreaks of disease identifiable by the appearance of chalkbrood mummies in brood cells or in front of the hive entrance were recorded throughout the year. For *Nosema* spp. infections, around 100 bees were sampled in spring and autumn (Fig. 1) as described previously (Gisder et al., 2010) and analysed microscopically followed by species differentiation via PCR-RFLP in positive samples (Table 2). In addition, clinical symptoms of nosemosis – if present – were recorded.

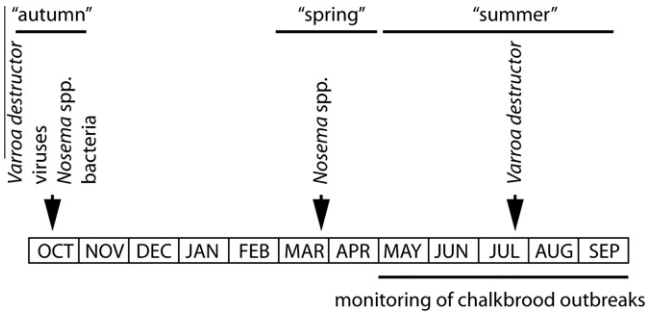


**Fig. 1.** Sampling scheme. One observation year started in October with collecting bees for determining the *V. destructor* autumn infestation level and the infection status in respect to viruses and *N. apis* and *N. ceranae*. In March, bees were collected for diagnosis of *Nosema* spp. infection in spring while the bees collected in July were analysed for the *V. destructor* summer infestation level. During the summer season, outbreaks of chalkbrood were recorded based on clinical symptoms (presence of chalkbrood mummies).

### 2.2. Nosema spp. detection and differentiation via PCR-RFLP

Qualitative diagnosis of *Nosema* spp. spores was performed by microscopic examination according to the method described in the "Manual of Standards for Diagnostics and Vaccines" published by the Office International des Epizooties (OIE), the World Organization for Animal Health (Anonymous, 2008) and as described recently (Gisder et al., 2010).

For *Nosema* spp. differentiation, microscopically positive homogenates were processed and analysed essentially as described recently (Gisder et al., 2010; Stevanovic et al., 2011). Briefly, differentiation via PCR-RFLP was performed by amplifying approximately 486 bp of the 16S rRNA gene in a PCR reaction using primers nos-16S-fw (5′-CGTAGACGCTATTCCCTAAGATT-3′, positions 22–44 in U97150; Gatehouse and Malone, 1999) and nos-16S-rv (5′-CTCCCAA CTATACAGTACACCTCATA-3′, positions 484–509 in U97150; Gatehouse and Malone, 1999). Subsequently, the PCR amplicons were subjected to restriction endonuclease digestions in two reactions at 37 °C for 3 h using *Msp* I/*Pac* I and Msp I/*Nde* I (New England Biolabs). The restriction endonuclease *Pac* I provides one unique digestion site for *N. ceranae* whilst the enzyme *Nde* I only digests *Nosema apis*. *Msp* I digests *N. apis* and *N. ceranae* and is used as a control for successful restriction digestion of PCR products.

### 2.3. Virus detection via RT-PCR

Virus detection was performed essentially as described recently (Genersch et al., 2010). Briefly, from each bee sample to be analysed