

ORIGINAL RESEARCH ARTICLE



Occurrence of parasites and pathogens in honey bee colonies used in a European genotype-environment interactions experiment

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Summary

Diseases are known to be one of the major contributors to colony losses. Within a Europe-wide experiment on genotype - environment interactions, an initial 621 colonies were set up and maintained from 2009 to 2012. The colonies were monitored to investigate the occurrence and levels of key pathogens. These included the mite *Varroa destructor* (mites per 10 g bees), *Nosema* spp. (spore loads and species determination), and viruses (presence/absence of acute bee paralysis virus (ABPV) and deformed wing virus (DWV)). Data from 2010 to the spring of 2011 are analysed in relation to the parameters: genotype, environment, and origin (local vs. non-local) of the colonies in the experiment. The relative importance of different pathogens as indicators of colony death within the experiment is compared. In addition, pathogen occurrence rates across the geographic locations are described.

La aparición de parásitos y patógenos en las colonias de abejas de la miel usadas en un experimento genotipo -medio ambiente europeo

Resumen

Es conocido que las enfermedades son uno de los principales contribuyentes a la pérdida de colonias. Como parte de un experimento a escala europea sobre la interacción genotipo - medio ambiente, se mantuvieron y supervisaron 621 colonias para investigar la incidencia, el nivel y la tendencia de los principales patógenos desde 2009 hasta 2012. Estos incluyen el ácaro *Varroa destructor* (ácaros por 10 g de abejas), *Nosema* spp. (carga de esporas y determinación de la especie) y virus (presencia / ausencia del virus de la parálisis aguda de las abejas (ABPV) y virus de las alas deformadas (DWV)). En un apiario se llevó a cabo un detallado estudio de casos, que también incluyó la cuantificación de los niveles de virus ABPV, DWV y el de las celdas de reinas negras (BQCV). Los resultados de las estimaciones de patógenos se analizaron en relación con parámetros como el genotipo, el medio ambiente, y el origen (local vs. no local) de las colonias en el experimento. Se comparó la importancia relativa de los diferentes patógenos como indicadores de la muerte de las colonias dentro del experimento. Además, se describen las tasas de incidencia de patógenos a través de las ubicaciones geográficas.

Keywords: COLOSS, Genotype-Environment Interactions experiment, honey bee, *Varroa destructor*, *Nosema* spp., viruses, survival

Introduction

Among the many factors that may have a role in honey bee (*Apis mellifera* L.) colony losses, diseases have been noticed for centuries, and were mentioned in the works of Aristotle (Creswell, 1862). One famous example for honey bee colony losses caused by disease was the "Isle of Wight Disease" in the beginning of the 20th century, which killed thousands of colonies in the British Isles (Imms, 1907) and was most probably caused by a combined infection with several pathogens interacting with adverse weather conditions (Ball and Bailey, 1991; Neumann and Carreck, 2010).

Fuelled by the recent and ongoing debate on worldwide honey bee decline and elevated colony losses, the role of pathogens has received intensive scientific attention in recent years (Tentcheva *et al.*, 2004; Chen *et al.*, 2006; Berthoud *et al.*, 2010; Genersch *et al.*, 2010; Vejsnæs *et al.*, 2010; Antunez *et al.*, 2012; Dainat *et al.*, 2012), providing a wealth of information on key pathogens linked to winter losses, but also to phenomena such as "Colony Collapse Disorder" (CCD) (Cox-Foster *et al.*, 2007; Cornman *et al.*, 2012; Nazzi *et al.*, 2012).

During the past four decades, the most serious threat for honey bees in the western world has been the invasive parasitic mite *Varroa destructor*, which has all but wiped out wild and feral honey bee populations and caused widespread damage to beekeeping in most of Europe, North America and New Zealand (Kraus and Page, 1995; Oldroyd, 1999; Todd *et al.*, 2007). Mite infestation of larvae, pupae and adult bees is known to cause immunosuppression (Yang and Cox-Foster, 2005, 2007), weight loss (Jong *et al.*, 1982), decreased flight performance (Duay *et al.*, 2002; Kralj and Fuchs, 2006), and reduction in lifespan (Schneider and Drescher, 1987; Amdam *et al.*, 2004). A serious *V. destructor* infestation leads to a diseased state of the colony, known as varroosis (Boecking and Genersch, 2008). In addition, varroa

mites act as vectors for viruses by facilitating their transfer between hosts (Ball, 1983; Bowen-Walker *et al.*, 1999; Chen *et al.*, 2004; Sumpter and Martin, 2004; Berthoud *et al.*, 2010). The mites have also been reported to activate inapparent virus infections in honey bees (Yang and Cox-Foster, 2005), probably by downregulation of honey bee immune genes (Nazzi *et al.*, 2012). Numerous studies have shown that *V. destructor* mediated viral infections can cause collapse of honey bee colonies, since viral titres are often strongly correlated with mite infestation levels (Ball and Bailey, 1991; Nordström *et al.*, 1999, 2003; Chen *et al.*, 2004; Shen *et al.*, 2005; Highfield *et al.*, 2009; Berthoud *et al.*, 2010; Dainat *et al.*, 2012; Francis *et al.*, 2013a).

In association with *V. destructor*, a complex of three closely related viruses (acute bee paralysis virus (ABPV), Kashmir bee virus (KBV) and Israeli acute paralysis virus (IAPV))(de Miranda *et al.*, 2010; 2013) have been reported to rapidly induce colony death (Genersch and Aubert, 2010; Francis *et al.*, 2013a). Deformed wing virus (DWV) is one of the most prevalent and commonly reported viruses in honey bees (Tentcheva *et al.*, 2004; Ellis and Munn, 2005; Gauthier *et al.*, 2007) and is strongly associated with *V. destructor* (Yue and Genersch, 2005; de Miranda *et al.*, 2013). The presence of the mite has been shown to increase prevalence of the virus while at the same time reducing its diversity by selecting for a single strain (Martin *et al.*, 2012).

Black queen cell virus (BQCV) (Bailey and Woods, 1977) is known to be widespread (Tentcheva *et al.*, 2004; Ravoet *et al.*, 2013; Lodesani *et al.*, 2014), but so far has not been suspected to play a major role in colony losses. It is not transmitted by *V. destructor*, but its association with the microsporidium *Nosema apis* (Bailey *et al.*, 1983) is well known. No reports exist to date on an association between the emerging parasite *Nosema ceranae* and BQCV, but such an association would seem likely, given the similar biology of the two *Nosema* species (Higes *et al.*, 2006; Klee *et al.*, 2007). Over the last few decades, *N. ceranae*

seems to have replaced *N. apis* in most parts of Europe (Klee *et al.*, 2007; Gajda *et al.*, 2013). *N. apis* has been linked to winter colony losses and poor spring build up (Fries, 1988), whereas *N. ceranae* has been reported to cause collapse of colonies in both spring and winter (Higes *et al.*, 2009). While infection with *N. ceranae* has been made responsible for symptoms very similar to those of CCD (Higes *et al.*, 2008), it has been more recently reported that its symptoms also may remain comparatively mild and need not necessarily cause colony losses (Paxton, 2010; Fernández *et al.*, 2012; Forsgren and Fries, 2012).

The main focus of most recent studies investigating the role of pathogens in colony losses has been on the pathogens, but comparatively little attention has been given to their host, the honey bee, or to interactions with environmental factors. Nonetheless, the genetic background of a honey bee colony may determine its susceptibility, or its resistance or tolerance to various pathogens such as *Paenibacillus larvae*, *Ascosphaera apis* and *Nosema* spp. (Behrens *et al.*, 2007; Jensen *et al.*, 2009; Huang *et al.*, 2013). The existence of several local strains of bee that have survived under *V. destructor* infestation pressure in the absence of treatment and which apparently possess strategies to limit mite population growth have been reported (Fries *et al.*, 2006; Le Conte *et al.*, 2007; Seeley, 2007), and “varroa tolerant” honey bee strains have been reported from several locations (Koeniger *et al.*, 1995; Kefuss *et al.*, 2004; Harbo and Harris, 2005; Rinderer *et al.*, 2010). However, it remains unclear whether such strains retain their tolerance when transferred into different environments.

Until now, there have been no studies which have investigated susceptibility, resistance or prevalence to diseases in honey bees with different genetic backgrounds and in different environments at the same time. The genotype-environment interactions experiment was run from summer 2009 to the spring of 2012 (Costa *et al.*, 2012) with the aim of understanding genotype-environment effects on survival and health status of honey bee colonies headed by queens originating from several different areas in Europe and tested in various locations under differing environmental conditions. In this paper, we report results from the experiment on the occurrence of *V. destructor* mites, *Nosema* spp. spores and several viruses. A complementary accompanying paper (Francis *et al.*, 2014a) reports detailed data from a case study at one of the sites (Chalkidiki, Greece) including a quantitative analysis of virus titers.

Material and methods

Experiment

The COLOSS genotype - environment interactions experiment was set up in the late summer of 2009 and ran until March 2012, starting with 621 colonies in 21 apiaries. The experimental design is described in detail in Costa *et al.* (2012), while Büchler *et al.* (2014) describe the survival of the colonies. A detailed map showing the distribution of genotypes at the experimental locations across Europe is shown in

Francis *et al.* (2014b). In the summer and autumn of 2009, new colonies were uniformly set up at each test location from package bees or brood nuclei, and queens of the different strains were introduced (for details, see Costa *et al.* (2012)). The experimental colonies received an initial treatment against *V. destructor* at the time of establishment, prior to 1 October 2009 (Büchler *et al.*, 2014). No chemical treatment against bee diseases was performed on any of the test colonies after this date, until the end of the experiment on 31 March 2012. The biological control measure of removing all brood at the peak of colony development in summer, followed by use of a trapping comb (Büchler and Meixner, 2008), could optionally be performed to reduce mite levels in colonies. When performed, brood removal had to be carried out uniformly in all colonies at any given apiary.

The colonies were managed according to a common protocol, which included colony inspections at regular intervals, and a continuous assessment of their health status. If colonies collapsed, the presumed reason of colony death was established by each apiary manager, based on previous observations together with specific samples collected at regular intervals (see details provided in Costa *et al.* (2012); Büchler *et al.* (2014)). Colony death was defined as “no further chance to survive by itself with regard to the local conditions”. Usually, colonies were considered “dead” when they had completely vanished. However, if the queen showed serious problems (e.g., drone layer) or the number of bees was too low to accomplish successful brood rearing, colonies were also regarded as lost and removed from the experiment. One apiary with 24 hives (Toulouse) could not be tracked until the end of the experiment, and in consequence was excluded from all evaluations. Therefore, all data presented in this paper are based on an initial number of 597 colonies at the beginning of the experiment.

In this paper, we report data on parasites and pathogens of the experimental colonies in the year 2010, since the data of 2011 and later were extremely unbalanced due to large amounts of missing data caused by collapsed colonies.

Sampling

Samples for *V. destructor* analysis consisted of at least 30 g of adult bees and were collected monthly, starting in June and ending in October or November (depending on the length of the season in each locality). Ten bees from the September or October sample (depending on location) were separated for analysis of viruses. Samples for *Nosema* spp. analysis consisted of at least 60 bees and were taken in the spring, summer and autumn of each year.

Samples for all disease analyses were collected from outer frames of the brood chamber or from a honey super (if present). *V. destructor* infestation in bees of the colony periphery has been shown to be more uniform than in the brood nest (Büchler *et al.*, 2010). For *Nosema* spp. analysis, the OIE guidelines (OIE, 2008) recommend sampling from the flight entrance, but our sampling was carried out during times without flight activity in several of the experimental apiaries. For this

reason, we agreed on a standard sampling from the colony periphery that was always possible. All samples were stored at -20°C until analysis.

Due to reasons of weather or experiment logistics (some apiaries were set up at remote locations), not all scheduled samples (varroa, nosema, viruses) could always be taken at all apiaries. In addition, not all partner institutions possessed the capacity of viral analysis and/or *Nosema* species determination. Therefore, these data are missing for some locations if analysis could not be achieved at a different institution.

Determining *V. destructor* infestation

After weighing each sample, the bees were soaked in detergent water for at least 30 minutes and shaken for at least one minute, to dislocate the mites from the bees (Costa *et al.*, 2012; Dietemann *et al.*, 2013). Bees and mites were then separated by straining, and the mites were counted. The number of mites per 10g of bees was calculated.

Virus analysis

The autumn samples (taken in September or October, depending on location) of 2010 were analysed for the honey bee viruses ABPV and DWV, known to be relevant for winter losses (Genersch *et al.*, 2010; Francis *et al.*, 2013a). Analogous to the procedure described in Genersch *et al.* (2010), total RNA was extracted from the heads of ten bees using standard methods (Yue *et al.*, 2006; de Miranda *et al.*, 2013). A one-step RT-PCR protocol was used to detect viral RNA. Primers and PCR conditions followed those of Genersch *et al.* (2010).

Nosema spp. infection level determination

The examination of samples for *Nosema* spp. presence and the level of infection were performed according to OIE guidelines (OIE, 2008; Fries *et al.*, 2013). The abdomens of 60 bees per sample were separated, homogenised in water and subsequently filtered through fabric with mesh size of 10 µm. The final volume of the macerate was then adjusted to 1 ml per bee with water. A drop of the suspension was examined in a counting chamber and the spores were counted.

Nosema species determination

Distinguishing between *N. ceranae* and *N. apis* was performed using spore suspensions (prepared as described in the paragraph above, from bees collected during early summer) previously used to define spore counts. DNA was extracted according to Fries *et al.* (2013) and PCR was carried out as described in Klee *et al.* (2007), OIE (2008) or as a species-specific qPCR as in Siede *et al.* (2008). Results obtained with the protocol and primers of Siede *et al.* (2008) were verified against those established with the method of Klee *et al.* (2007). Results obtained with the OIE (2008) method were compared against results achieved with the method published in Gisder and Genersch (2013). No differences were found in either case

Statistical methods

V. destructor infestation data were log-transformed to correct for non-normal distribution and to reduce the interclass variation. The mite infestation levels in the autumn of 2010 were examined using a GLM analysis with location and genotype, origin of breed (local vs. non-local) and brood removal as fixed factors. Also, in order to examine the influence of infestation level on colony survival for each sampling month (June to October in 2009, 2010 and 2011) and each location, the median infestation value was calculated and a binary logistic regression analysis was carried out. Further on, a survival curve (Cox, 1972) comparing survival duration of colonies below and above the location-specific infestation median was calculated. The survival model assumes that the individual hazard function depends on a common baseline hazard and the values of the covariates. The ratio of the estimated hazards over time is supposed to be constant for any two colonies with particular values for time-independent covariates.

The results of qualitative virus analysis (ABPV and DWV) were available as binary data (presence or absence). The virus presence was analysed with a GLM procedure with location, genotype and local vs. non-local origin as fixed factors, and the log-transformed *V. destructor* infestation as covariable.

To facilitate statistical analysis, *Nosema* spp. spore counts per bee were grouped into four categories, corresponding to no infection, low, medium and high levels of infection (0: no spores, 1: below 1 million, 2: between 1 and 10 million, and 3: above 10 million). A GLM procedure was carried out including the fixed factors season, location, genotype and local vs. non-local origin. Average *Nosema* spp. spore levels in Northern and Southern Europe were compared using a Wilcoxon test. The northern group consisted of Finland, Denmark, the three sites in Poland, the three sites in Germany, Austria, and one of the Macedonian sites (Bitola), while the southern group contained the apiaries in Croatia, Italy, Bulgaria, Greece, and the two remaining Macedonian sites (see also Hatjina *et al.* (2014)).

All statistical analyses were carried out using R software 3.0.1 (R Development Core Team, 2008), or SPSS 20.0 (IBM, 2011).

Results

V. destructor

Of the initial 597 colonies, 503 died before the end of the experiment in March 2012 (Büchler *et al.*, 2014). In 192 of these (38%), *V. destructor* mite load was reported as the primary cause of colony collapse, based on observations by the apiary manager or on sample analysis. Thus, varroa was the main cause for colony death, followed by "queen problems" (16.9%), and "nosema" (7.3%). Various other reasons, such as starvation, weakness or robbing were pooled under

“other” (33.8%) (see also Büchler *et al.*, 2014). With the exception of the location Probistip (Macedonia) where all colonies died within six months from the start of the experiment, colony losses due to varroa infestation were only observed after approximately one year.

Huge variation was observed in the *V. destructor* infestation levels of the autumn 2010 at different experimental locations. With the exception of the southernmost locations (Termini Imerese, Chalkidiki) the colonies were free of brood at the time of this measurement (see also Hatjina *et al.*, 2014). The differences in mite loads between locations greatly exceeded the difference between mite loads of colonies that survived or collapsed in the following winter (2010/2011, approximately 500 days into the experiment) at a given location (Fig. 1.).

A GLM analysis shows that the autumn *V. destructor* infestation level in 2010 was most strongly influenced by location. There was no significant difference between the mite infestation level of colonies of different genotypes or those headed by queens of local *vs.* non-local origin (Table 1). For each location and each sampling month, the median infestation value was calculated and a binary logistic regression was carried out, in which the fate of colonies with infestation rates below and above the location-specific median was compared. In this analysis, genotype showed a significant effect ($F = 3.153$, $p < 0.0001$), but the differences between local and non-local bees were not significant (see Online Supplementary Table S1 for complete binary logistic results table). [<http://www.ibra.org.uk/downloads/20140508/download>]

Comparing the colonies with mite levels below and above the location-specific median in a survival analysis (Büchler *et al.*, 2014) resulted in a significant difference of survival time. At the end of the experiment 22.8% of the colonies with below median infestation levels were still alive, compared to only 15.2% with above median infestation levels (Fig. 2.). The complete analysis is given in Table S1.

Virus infections

The proportion of ABPV and DWV in the autumn of 2010 was highly significantly different between locations and genotypes (Figs 3A.,B.). The site in Finland, one site in Macedonia (Skopje) and the three Polish locations had a very low (or zero) rate of positive samples. In contrast, in Bulgaria and Sicily a very high or 100% rate of double infections was observed. High ABPV incidence rates of 80% and 100% were only observed in two environmental clusters with high average temperatures (Termini Imerese, Unije, Chalkidiki, and Bulgaria, respectively), while occurrence in all other clusters remained below 20% (for definition of clusters, see Hatjina *et al.* (2014)). Among the genotypes, LigF and MelP were free of virus. There were no differences in virus incidence between native and non-native genotypes.

In general, DWV was more common than ABPV. In the second year (2011) we observed a tendency to decreasing ABPV and increasing DWV, but the data are too sparse for analysis (many colonies collapsed).

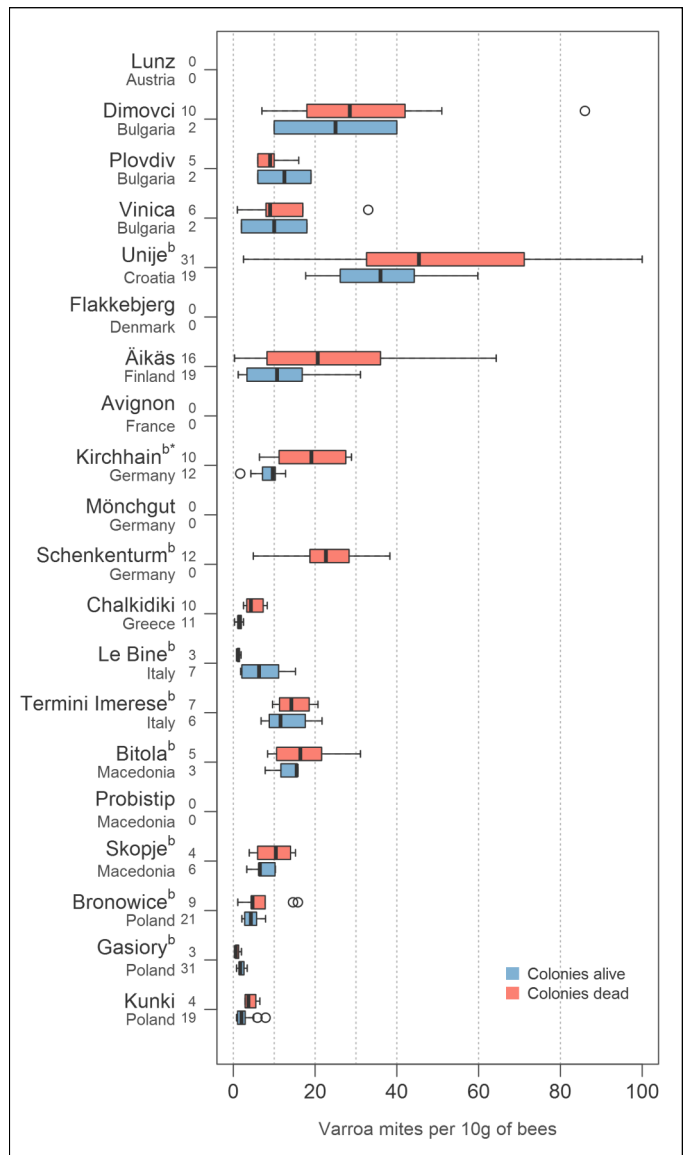


Fig. 1. Boxplot showing varroa mites per 10 g of bees in colonies during autumn 2010. The dark vertical bar denotes the median. The open circles denote outliers. Colonies are categorised into 'Alive' or 'Dead' based on their status in spring 2011. The number of colonies sampled in each category is indicated. Zero indicates that no colonies were sampled. The superscript letter 'b' on the location name indicates that brood removal was carried out at that location. At locations marked with an asterisk (*), the difference in mite levels between survivors and non-survivors was significant.

GLM analyses show that the presence of DWV (Table 2) and ABPV (Table 3) in the autumn of 2010 was significantly influenced by location, but not by genotype. There was also no difference in virus occurrence between local and non-local bees. We found no significant contribution of the autumn infestation level of *V. destructor* to the occurrence rate of ABPV or DWV.

Table 1. GLM analysis of Varroa infestation (\log_{10} -transformed) in autumn 2010.

Dependent variable: Varroa autumn 2010 (\log_{10})					
Source	Type III Sum of Squares	df	Mean square	F	Sig.
model	327.092 ^a	32	10.222	127.350	0.000
location	38.253	14	2.732	34.042	0.000
genotype	1.049	15	0.070	0.871	0.597
brood removal	0.012	1	0.012	0.150	0.699
local vs. non-local	0.044	1	0.044	0.552	0.458
error	21.109	263	0.080		
total	348.201	295			
a. R-squared = 0.939 (adjusted R-squared = 0.932)					

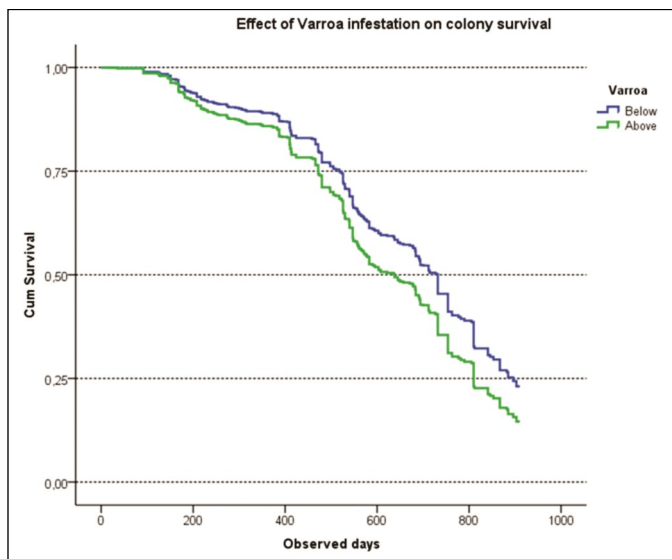


Fig. 2. Trajectory of colony survival for colonies with lower and higher infestation level than the median at their respective location. X-axis: duration of experiment (days), Y-axis: proportion of colonies alive. Below: colonies with Varroa infestation (mites /10g bees) below the median infestation level at their location; above: colonies with Varroa infestation above their location-specific median.

***Nosema* spp.**

Colony losses reported to be caused by *Nosema* spp. ($n = 37$) occurred only in the test apiaries Le Bine (Italy), Chalkidiki (Greece), Äikäs (Finland) and Mönchgut (Germany). Losses at the Italian site occurred very early in the experiment and accounted for the majority (25 of 37) of all losses caused by *Nosema* spp. In the spring of 2010, at five locations, no *Nosema* spp. at all were observed, and at eight more sites the majority of colonies were also free of *Nosema* spp. In contrast, at two of the Polish sites (Kunki and Gąsior) *Nosema* spp. were present in more than 80% of the colonies, with levels above 1 million spores per bee in more than 50% of the colonies (Fig. 4.). An analysis of distribution in the different genotypes is not possible, since there are too many missing data across locations. However, we observed that colonies of *A. m. ligustica* (genotypes LigI and LigF) were present in three of the four locations where losses due to *Nosema* spp. occurred.

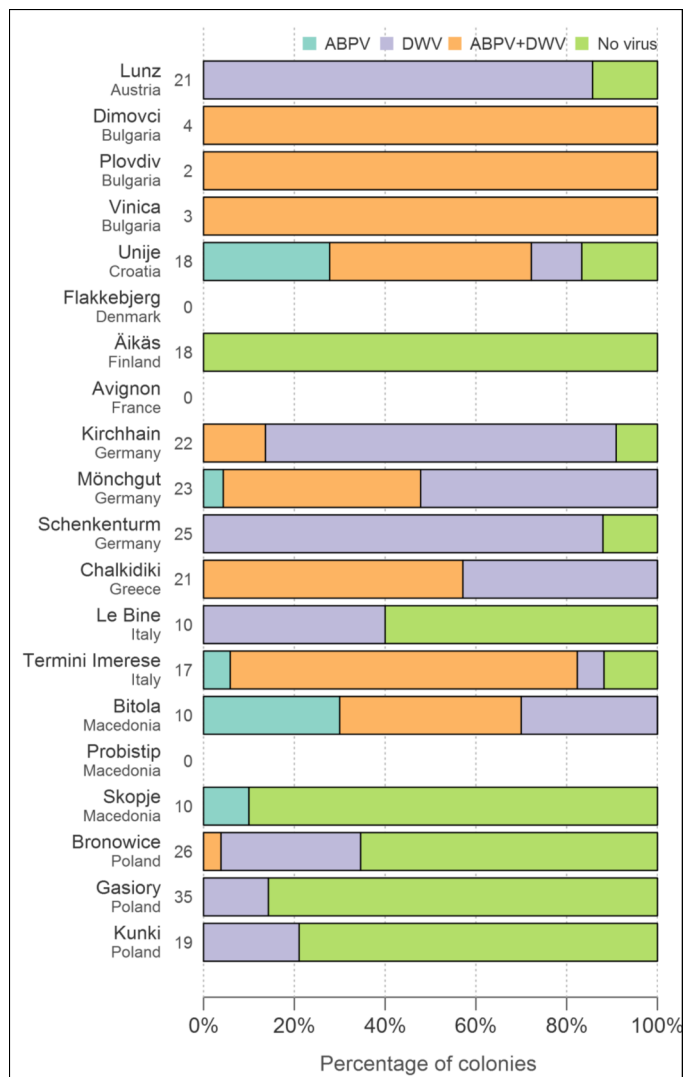


Fig. 3a. Incidence rate of single and double infections with ABPV and DWV in the different locations (autumn 2010). The number of colonies sampled at each location is indicated.

Nosema spp. infection levels (expressed in classes 0 to 3) were significantly different between seasons, with significantly lower infection levels in summer compared to spring and autumn ($F = 43.005$, $p < 0.05$, Bonferroni-corrected) and locations ($F = 8.893$, $p < 0.05$, Bonferroni-corrected) (Fig. 4.), but not significantly different between local and

Table 2. GLM analysis of DWV in autumn of 2010.

Dependent Variable: presence of DWV autumn 2010					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	83.346a	32	2.605	20.925	0.000
location	16.133	14	1.152	9.258	0.000
genotype	1.966	15	0.131	1.053	0.403
Local vs. non-local	.453	1	0.453	3.639	0.058
Varroa autumn201(log ₁₀)	.138	1	0.138	1.108	0.294
Error	22.654	182	0.124		
Total	106.000	214			
a. R Squared = 0.786 (Adjusted R Squared = 0.749)					

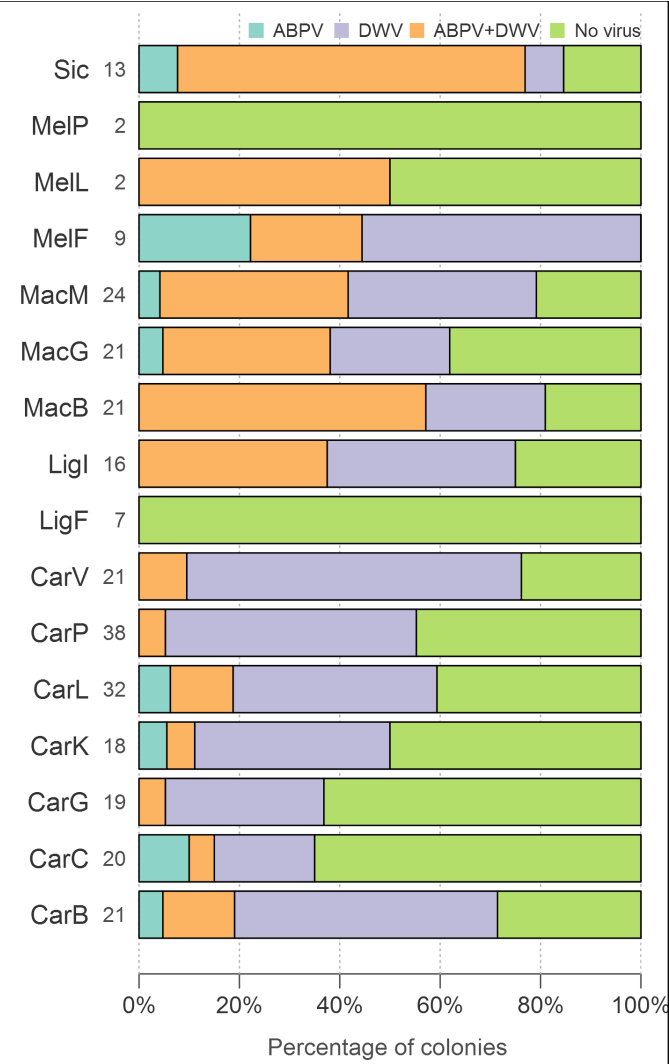


Fig. 3b. Incidence rate of single and double infections with ABPV and DWV in the different genotypes (autumn 2010). The number of colonies sampled for each genotype is indicated.

non-local genotypes. No significant difference was observed between colonies that survived until the end of the experiment and colonies that collapsed before. Differences in infection levels were significant (Wilcoxon, $P < 0.001$) between apiaries in Northern and Southern Europe, where Northern colonies had higher spore counts than the Southern colonies.

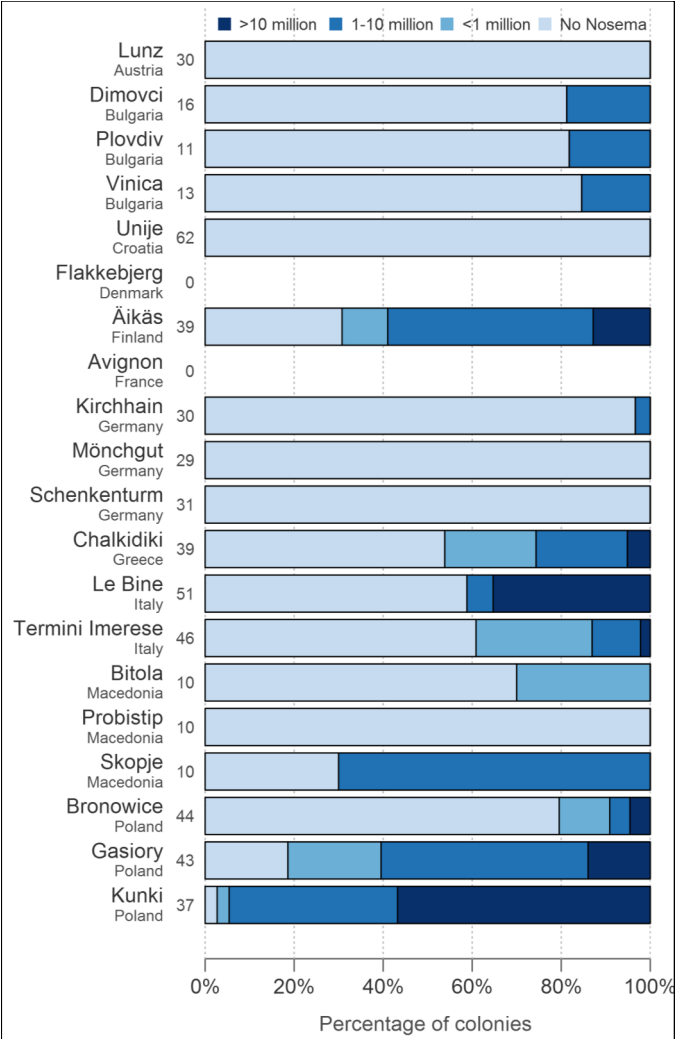
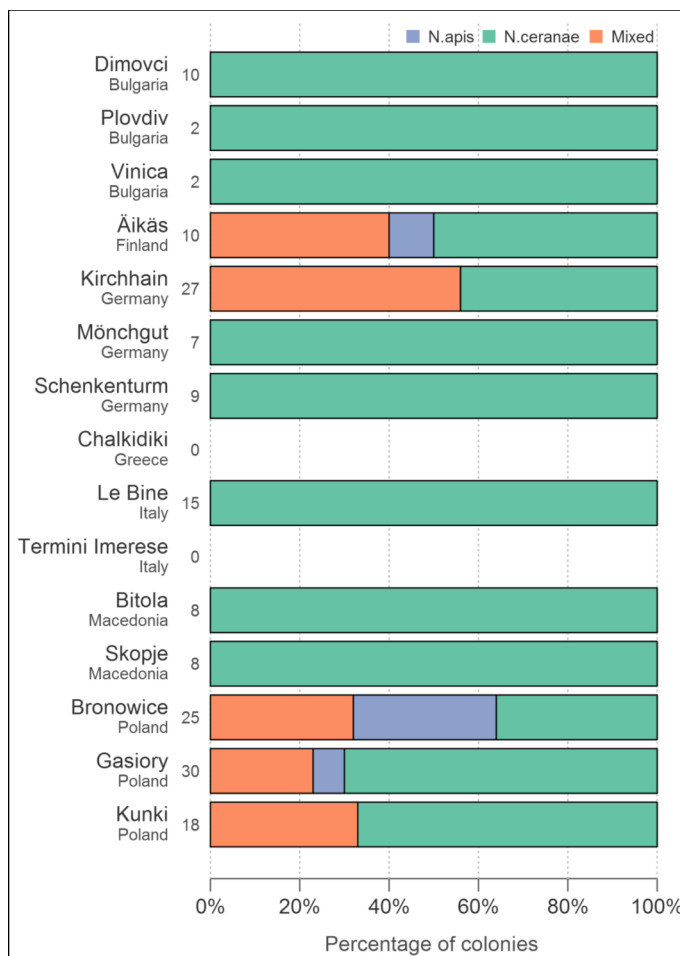


Fig. 4. *Nosema* spp. levels across locations in spring 2010. The number of colonies sampled at each location is indicated.

The distribution of *N. apis* and *N. ceranae* across our experimental locations is shown in Fig. 5. In most apiaries analysed, only *N. ceranae* was observed. *N. apis* only occurred in a few Northern European sites and was predominantly found in mixed infections. Pure *N. apis* infections were only observed in Finland and in two apiaries in Poland.

Table 3. GLM analysis of ABPV in autumn of 2010.

Dependent Variable: presence of ABPV autumn2010					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	38.957a	32	1.217	14.729	0.000
location	10.995	14	0.785	9.502	0.000
genotype	0.591	15	0.039	0.477	0.950
Local vs. non-local	0.134	1	0.134	1.619	0.205
Varroa autumn2010 (log₁₀)	0.021	1	0.021	0.251	0.617
Error	15.043	182	0.083		
Total	54.000	214			
a. R Squared = 0.721 (Adjusted R Squared = 0.672)					

**Fig. 5.** Distribution of *Nosema* spp. across the experimental locations in 2010. The number of colonies sampled at each location is indicated.

Discussion

In this paper, we describe observations on the pathogen and parasite status of honey bee colonies in a European Genotype - Environment interactions experiment. A starting number of 597 colonies were followed for a period of two and a half years. During the experiment, no chemical treatment against bee diseases was applied. As colonies succumbed, the primary cause of mortality was established by each apiary manager, based on previous observations together with specific samples collected at regular intervals.

The fate of untreated colonies over several years has been followed and described in numerous previous studies (Berg *et al.*, 2001; Büchler *et al.*, 2002; Kefuss *et al.*, 2004; Fries *et al.*, 2006; Le Conte *et al.*, 2007); although we had to restrict the analysis of most of our data to the first year of the experiment, our study is the first one to present comparative survival and *V. destructor* infestation data on a European scale, and to include analyses of other honey bee pathogens such as viruses and *Nosema* spp. In addition to examining the colonies for the occurrence of notorious parasites and pathogens, we also analysed the resulting data for effects of colony genotype and environment, and for interactions between these two factors. The analysis of colony pathogen data within the GEI experiment resulted in a strong overall predominance of location influencing disease prevalence. For all pathogens analysed, the environmental component either was the only significant factor, or, if a genotype component was present, the environmental influence was stronger.

The *V. destructor* infestation levels in the autumn of 2010 between the experimental locations differed immensely, where the differences between apiaries by far exceeded the difference between surviving and collapsing colonies at each location. Nonetheless, our survival analysis (Fig. 2.) clearly demonstrates that survival at each location significantly depended on the relative *V. destructor* infestation level, since colonies with more mites were more likely to collapse.

Several publications report variability of *V. destructor* population dynamics in response to environmental conditions, such as length of the brood season and bee colony density (Ritter, 1984; Martin, 2001; Le Conte *et al.*, 2010; Rosenkranz *et al.*, 2010). Our experiment was carried out under widely differing climatic and environmental conditions that have been demonstrated to influence colony size, brood rearing and productivity of the genotypes in the test (Hatjina *et al.*, 2014). In consequence, all these parameters have an effect on mite reproduction and quite possibly also on the mite infestation level a colony is able to tolerate.

For example, autumn infestation levels observed at the Unije site, but also in Dimovci (Bulgaria) were extremely high, but are in the range of those observed during the previous population comparison experiment on Unije between 2000 and 2004 (Büchler *et al.*, unpublished; Büchler *et al.*, 2002). In our experiment, mite invasion from collapsing colonies

with extreme mite levels to their neighbours was prevented by continuously monitoring colony strength and mite infestation so that colonies in danger of collapsing could be identified and treated or removed from the apiary in time. At the same time, they were considered “dead” and excluded from further analyses.

Comparatively few reports describe mite levels considered responsible for colony damage or death from different environments (Delaplane and Hood, 1999; Strange and Sheppard, 2001; Currie and Gatién, 2006; Büchler *et al.*, 2010; Carreck *et al.*, 2010; Genersch *et al.*, 2010). The results presented here indicate that threshold levels for colony damage and death are very likely to differ substantially across Europe. As detailed by Hatjina *et al.* (2014), the population dynamics of colonies differs significantly between the north and south of Europe. The ratio of adult bees to brood is much lower in the south, and the life expectancy of bees in the south is generally shorter than in the north. It is possible that an additional life-shortening effect of *V. destructor* infestation has less dramatic consequences in south European climates. Our data, while insufficient to permit a differentiated determination of thresholds for each region, give preliminary indications of considerable differences across Europe. The existing threshold data for Germany (Büchler *et al.*, 2010; Genersch *et al.*, 2010) have been compiled from several years of continuous observation of thousands of colonies.

Certain aspects of honey bee colony behaviour, such as swarming and hygienic behaviour are known to influence the *V. destructor* infestation level of a colony throughout the season. Within the GEI experiment, the expression of these behaviours was continuously observed and is detailed in a separate publication (Uzunov *et al.*, 2014). Swarming affects *V. destructor* reproduction due to the interruption of bee brood production, although the results of Fries *et al.* (2003) demonstrated that swarming did not have a significant effect on the build up of detrimental mite levels. However, as mites leave on the departing bees (Wilde *et al.*, 2005) the fission of the colony results in a reduction of mite levels. It appears likely that the swarming behaviour of the colonies in our experiment influenced the mite levels, but the structure of our data does not allow an in-depth analysis. Beyond genetic background and climate, other factors, such as age of the queens appeared to also influence swarming behaviour. For example, the tendency to swarm was much more pronounced in the second year of the experiment (Uzunov *et al.*, 2014). In addition, apicultural measures (such as temporary colony splits) were applied to prevent colonies from swarming, and therefore the full impact of the undisturbed behaviour could not be measured within the context of this experiment.

To untangle the complex interactions between genetic background, environment and factors related to the status of the colony itself, controlled experiments with known start levels of infestation will be required. It should be noted that our study had a predominantly observational character and its ultimate aim was to investigate the

effects of genotype and environment on colony survival in the absence of chemical control of *V. destructor* (Büchler *et al.*, 2014).

Our analyses identified a highly significant influence of the apiary location on the occurrence of both ABPV and DWV. At a few experimental sites (Finland, Skopje (Macedonia), and the three Polish sites) the incidence rates of DWV and ABPV were very low or even zero during the course of the experiment. Likewise, mite levels in Poland and Skopje also remained comparatively low during the entire observation period. Since the two viruses in question are known to be vectored by and, in the case of DWV, to also replicate in mites (Ball, 1983 1989; Boncristiani *et al.*, 2009), the low mite infestation may have contributed to a low or undetectable level of virus infections. The distribution of ABPV was particularly remarkable, since high incidence was only observed at locations of two environmental clusters with high average annual temperatures, and, correspondingly, a long or medium active season (Hatjina *et al.*, 2014). These results have to be interpreted with caution, since our observation and sampling strategies were not optimised and probably carry a considerable non-detection error. Nonetheless, Topolska *et al.* (2009) also report significantly lower ABPV infection rates from colder regions of Poland which may reflect an influence of climatic conditions to the distribution of this virus. However, it remains difficult to separate a direct effect on virus propagation from the influence of climate on *V. destructor* reproduction, and additional data from continuous observations of colonies across different climates will be needed to confirm this trend.

Unexpectedly, we did not observe a statistically significant influence of mite infestation levels on the occurrence of ABPV and DWV, although both viruses are known to be associated with and transmitted by *V. destructor* (Ball and Allen, 1988; Bowen-Walker *et al.*, 1999; Chen *et al.*, 2004). Several factors may have contributed to this. First, the variation in virus occurrence was extremely high, where especially DWV was also found to be present in colonies with comparatively low mite levels. Secondly, the statistically significant uneven distribution across geographic locations may have interfered with detection of the association with *V. destructor*. The fact that we used the comparatively insensitive method of endpoint PCR on only ten bees per colony probably also contributed, since it may have produced a number of false negative results. This is rendered likely by the fact that we find a clear correlation between mite infestation levels and viral titres of ABPV and DWV in the detailed case study on the apiary of Chalkidiki (Francis *et al.*, 2014a), where qPCR analysis, combined with a quantification of virus copies was used.

At the site in Finland, we observed a high infestation rate of 20 mites per 10 g bees and more also from surviving colonies, but no virus was detected in any of the colonies during the entire experiment. These infestation rates correspond to mite levels reported from Germany in the 1980s without causing visible symptoms (Boecking and Genersch,

2008) and appears to support the hypothesis that *V. destructor* infestation is less detrimental to bee colonies in the absence of DWV or ABPV (Carreck *et al.*, 2010).

Nosema spp. spore levels were significantly and most strongly dependent on location, where colonies stationed in apiaries in Northern Europe had higher spore numbers than the ones from Southern Europe. This observation is consistent with the hypothesis that *Nosema* spp. spore levels generally increase when bees are confined, such as in the autumn and winter in colder climates (OIE, 2008). This is also reflected in the observation that differences between seasons were significant across locations, with higher spore levels occurring in autumn and spring.

According to the records taken during the experiment, *Nosema* spp. contributed to colony losses in only four apiaries (Chalkidiki, Le Bine, Mönchgut, and Äikäs). In the remaining apiaries there was no difference in *Nosema* spp. spore levels in colonies that died and in those which survived until the end of the experiment, suggesting that *Nosema* spp. infections did not play a major role in colony losses. The difference in distribution of individual *Nosema* species lends support to the hypothesis that in warmer climates *N. ceranae* is more competitive than *N. apis* (Gisder *et al.*, 2010; Forsgren and Fries, 2012). Our data on *Nosema* spp. infection levels and *Nosema* species distribution do not support the hypothesis that *N. ceranae* has to be regarded as a major driver of colony losses (Higes *et al.*, 2006, 2008, 2009).

The aim of this paper is to describe honey bee health in an experiment on colony survival in the absence of chemical control of *V. destructor* that involved different genotypes of bees in different environments across Europe. It was not our intention, and our data are not suited to explore effects of specific pathogens on specific genotypes or under certain environmental conditions. The pathogens we recorded were natural infections, probably widespread in the neighbourhood of each apiary. Therefore, our results do not provide any basis for an analysis of differences in susceptibility between subspecies or genotypes. The infectious nature of pathogens, combined with the high density of colonies within apiaries, and short distances between individual colonies of different genetic background, will affect the apiary outcome in a non-stochastic fashion. Therefore, we caution against drawing general conclusions from our results, as to the performance of the various subspecies in the experiment. Rather, we envisage the data presented here as a confirmation that neither the occurrence nor the absence of a particular pathogen can explain the main outcome of the experiment on honey bee colony survival.

In summary, our results clearly demonstrate that the location of an apiary has a significant and strong effect on the presence of any pathogen in its colonies. In particular, colonies headed by queens descending from “survivor stock” (Le Conte *et al.*, 2007) or from

“varroa-tolerance” selection programmes (Büchler *et al.*, 2010) did not show an overall improved survivorship or resistance to *V. destructor*.

Likewise, no significant differences in disease occurrence between local and non-local experimental colonies were observed. Clearly, both local and non-local honey bees become infected and suffer from diseases. Yet, the results of the survival experiment unambiguously indicate a better survival of local genotypes in comparison to introduced ones, as detailed in Büchler *et al.* (2014). However, the pathogen case study (Francis *et al.*, 2014a) indicates that the level of pathogens in colonies of non-local origin is generally higher, which may be the result of unsatisfactory adaptation to the local environment which may consume resources local bees can apply to keep pathogens in check. Among the factors contributing to the differences between local and non-local bees in coping with *V. destructor* and the accompanying virus infections may be variation in the geographic distribution of virus strains. For several viruses, considerable genetic variation has been shown (Cornman *et al.*, 2013). Recent research identified a selective sweep among strains of DWV after the arrival of *V. destructor* in a previously mite-free population of honey bees (Martin *et al.*, 2012) selecting for a single, presumably more virulent strain of virus. It may be possible that non-local bees possess even fewer resources to cope with such strains than locally adapted bees that have been continuously exposed to these pathogens.

The data collected in this experiment also permit a preliminary view of disease presence in several European countries. With a few exceptions in single countries (Topolska *et al.*, 2009; Genersch *et al.*, 2010), there are no recent comprehensive compilations of honey bee pathogen presence in Europe. However, the currently ongoing pilot surveillance project on honey bee colony losses, coordinated by the European Reference laboratory for honey bee diseases, may provide an improved view in due course (Chauzat *et al.*, 2013a, 2013b).

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