Apidologie
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DOI: 10.1007/s13592-013-0201-1

Original article

Negative evidence for effects of genetic origin of bees on *Nosema ceranae*, positive evidence for effects of *Nosema ceranae* on bees

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Received 21 September 2012 – Revised 3 January 2013 – Accepted 29 January 2013

Abstract – In two tests, honey bee colonies of different origins were sampled monthly to detect possible differential infection with *Nosema ceranae*; colony sizes and queen status were monitored quarterly. One experiment used queens crossed with drones of the same type obtained from colonies which had previously exhibited high and low infections. A second experiment used queens from ten commercial sources. No clear genotypic (P=0.682) or phenotypic (P=0.623) differences in infection were evident. Colony deaths and supersedures did not relate significantly with infection except for deaths of colonies in the autumn (P=0.02). Significant effects on colony growth were found in all seasons: average 3-month decreases in population ranged from 0.4 to 1.4 frames of bees per million *N. ceranae* per bee. These results confirm that *N. ceranae* can be involved in weakening of colonies even in warm climates and suggest that breeding for resistance may require more intense selection, larger base populations, or different screening methods.

***Nosema ceranae* / *Apis mellifera* / honey bees / resistance / breeding / selection**

1. INTRODUCTION

Nosema ceranae is a honey bee (*Apis mellifera*) parasite that has expanded its distribution in the last decade (Klee et al. 2007; Chen et al. 2008) and adversely affects bee health. Worker bees maintained in cages exhibit compromised immune function (Antúnez et al. 2009; Chaimanee et al. 2012) and disruptions of carbohydrate metabolism (Mayack and Naug 2009, 2010) within a week after ingesting spores. Additionally, the mortality of highly infected caged workers increases dramatically after the first week and most workers are dead within 3 weeks (Higes et al. 2007). In field colonies, the prevalence of highly infected bees is generally low (Bourgeois et al. 2012; Smart and Sheppard 2012). However, under some conditions, untreated colonies may collapse when prevalence is above 50 % (Higes et al. 2008).

Negative effects on field colonies appear to be variable and not as predictable as effects on infected individual workers. Recent studies have not found an influence of *N. ceranae* levels on colony overwintering mortality (Genersch et al. 2010; Guzmán-Novoa et al. 2010; Williams et al. 2010) or on population sizes of colonies after overwintering (Williams et al. 2010) or in the summer (Traver and Fell 2011). In contrast, colonies with higher spore loads had poorer development during periods of colony growth (Higes et al. 2008; Guzmán-Novoa et al. 2010; Eischen et al. 2012). Subtle effects beyond outright mortality and weaken-

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ing may occur given that populous colonies surviving with moderate to high levels of infection have reduced pollination abilities (Anderson and Giacon 1992; Eischen et al. 2010).

These possible risks are being addressed with extensive prophylactic treatments that rely primarily on the antibiotic fumagillin. Development of bees with genetic resistance could be the basis of a more sustainable management program for *N. ceranae*, but this approach has not been explored rigorously. Several studies suggest that it may be possible to find useful levels of genetic resistance to *N. ceranae* by screening colonies of diverse origins. Phenotypic variation has been documented (Rinderer and Sylvester 1978; Malone et al. 1995; Malone and Stefanovic 1999) among strains and colonies of honey bees for susceptibility to *N. apis*, the species that was predominant in North America until recently. Bee breeders in Denmark are reported to have improved resistance to *Nosema* spp. in their breeding stocks by culling colonies that show any *Nosema* spores in the spring (Traynor and Traynor 2008), although the improvement may not be based on decreased infection but rather stronger immune responses to infection (Huang et al. 2012). Patriline differences in infection with *N. ceranae* within the same colony reveal a genetic component to infection (Bourgeois et al. 2012).

We sought evidence of genetically based resistance of *A. mellifera* to *N. ceranae* by measuring the natural infections of monthly samples taken from field colonies that had queens of different origins. In one experiment we used queens produced by us from colonies that differed in infection during an initial screening. In a second experiment, we compared possible phenotypic differences in infection of groups of colonies with queens obtained from diverse origins and geographic locations. Measurements of bee populations, queen status and survival of colonies in both experiments allowed quantification of possible effects on colony performance from the observed levels of *N. ceranae*.

2. MATERIALS AND METHODS

2.1. Experiment 1—propagation and evaluation of high- and low-infection field colonies

An initial group of 24 colonies was established in Baton Rouge, Louisiana in November 2009 by installing packages with 1.25–1.5 kg bees taken from a large mix of bees shaken from infected colonies (Harbo and Hoopingarner 1997). No treatments for *Nosema* spp. had been applied to the colonies in the prior year; infection of 3 100-bee samples from the original mix of bees used to make the packages ranged from 0.8 to 1.5 million individual *N. ceranae* per bee (our molecular assay detects both vegetative cells and spores; see Bourgeois et al. 2010 for further explanation). The packages received queens of diverse genetic origins (Russian, VSH, commercial Italian, and specifically provided by two beekeepers in Alabama and in Washington state who reported improved performance in the presence of *N. ceranae*). Packages were established in November in hive equipment with sufficient honey stores for overwintering and were managed following typical procedures for the area, but did not receive supplemental feeding or treatments for pathogens or mites. In May, we chose viable colonies which had the lowest (L, n=5) and highest (H, n=5) average monthly infections from January to April (ranges: L=0.4−2.9 and H=5.0−10.9 million *N. ceranae* per bee). Daughter queens from the five H and five L colonies were instrumentally inseminated with multiple drones from two and five colonies of their same types (H X H and L X L; three H colonies did not produce drones).

H and L queens were introduced into queenless divisions of the parent colonies. Colonies were managed as required by the season (except for not receiving treatments or supplemental feeding) from May 2010 until April 2011. In June, August, and November 2010, and in February and April 2011, we recorded queen status, general condition of the colony, and colony population as number of frames at least two thirds covered with adult bees. Colonies in which the original queen was superseded were not tracked further.

2.2. Experiment 2—colonies with commercial queens

From May to August 2010, 12 to 15 queens from each of 10 diverse commercial sources were introduced into queenless divisions of untreated colonies in a stationary commercial beekeeping apiary in Port Barre, Louisiana. Queens obtained from the southern USA (S1-6) and imported from Australia were introduced into colony divisions from May to June 2010. Queens from northern sources (N1-3, including one from Canada) were introduced in July and August 2010. Parent colonies in the apiary had not been treated with fumagillin, and had active but variable *N. ceranae* infections. Each division was sampled at the time of queen introduction. Colonies were managed by the beekeeper following typical commercial procedures for the area except that no treatment was used against *Nosema* spp. Colonies were fed and were treated for *Varroa destructor*, *Paenibacillus larvae*, and *Aethina tumida* in the autumn and early spring. Colony conditions were measured as in Experiment 1.

2.3. Measurements of *N. ceranae* and analyses of colony infection

Samples of approximately 100 workers were taken each month from each colony with an original queen from lids or honey combs furthest away from brood areas and frozen at −20 °C until further processing. One hundred bees were counted, ground in a Sterling Multimixer (Sterling, Inc.) with stainless steel cups modified to improve tissue disruption and then processed following procedures as previously described (Bourgeois et al. 2010) except that only *N. ceranae*-specific primers were used to obtain the number of *N. ceranae* individuals per bee. The conversion from *N. ceranae* individuals estimated with this technique to spore counts using a haemocytometer is a factor of 0.25 spores per total number of *N. ceranae* (Bourgeois et al. 2010). Monthly estimates of infection (in millions *N. ceranae* individuals per 100 workers) for each surviving colony with an original queen were compared between H and L colonies (Experiment 1) and between colonies of the ten commercial sources (Experiment 2) as separate repeated measures ANOVA on $\log_{10}$-transformed variables. For Experiment 1, an additional categorical random variable (source colony that was divided to produce the queenless unit) and a covariate (the initial infection of the queenless unit) were included in the analyses initially and were removed when non-significant. Data from Australian queens were not included in analyses for Experiment 2 due to lack of colonies with original queens for the last 3 months of the experiment. Least squares means from the analyses for both experiments were transformed back to the original scale for presentation. Least significant ratios between pairs of means on the original scale were derived from least significant differences generated from the analyses of log-transformed means.

2.4. Analyses of effects of infection on colony conditions

Monthly samples for colony infection, and seasonal observations of colony condition and population for the two experiments allowed us to track when a colony died, when the original marked and clipped queen was superseded (within a 2- or 3-month period), and changes in populations through different seasons. A change in bee population (final–initial frames of bees in each time period) was calculated for each of the 3-month periods ending in August, November, February, and April (except for a 2-month period February to April). In addition, the average infection for each colony was calculated for each time period.

Maximum likelihood estimates of the logistic function were used to calculate the significance of the probability of death and the probability of supersedure related to the average infection of each colony for the period ending at the time of the observation. For colonies that did not die or supersede, the effects of apiary (Experiment 1 vs. Experiment 2 as categorical factors) and infection (as a linear regressor) on change in colony population were calculated with analysis of covariance. The effect of infection on population change was estimated by the slope (β) of the relationship between *N. ceranae* infection and population change. Additionally, the relative contributions (R^2) of apiary and of *N. ceranae* infection, combined or as individual or factors, to the total variation were calculated for each of the four time periods.

3. RESULTS

3.1. Experiment 1

Colonies propagated from H and L parents did not differ in their average infection ($F_{1,\ 27}$= 0.17, P=0.682), while the month of sampling had a strong effect ($F_{9,\ 203}$=17.97, P<0.001; Figure 1). There was no significant interaction between colonies of the two types and month of sampling ($F_{9,\ 203}$=0.79, P=0.626). Ratios of average infections in H and L colonies in four of the 10 months were opposite to an expected ratio of >1. Only in March 2011 was the ratio of infection between H and L colonies close to significance (ratio=19 vs. least significant ratio=27).

3.2. Experiment 2

The infection of colonies was significantly influenced by month ($F_{8,\ 247}$=10.00, P<0.001; Figure 2), but not by commercial bee source ($F_{8,\ 47}$=0.78, P=0.623), and source and month did not interact ($F_{64,\ 247}$=0.99, P=0.513). Estimated least significant ratios (at P<0.05) between average infections of pairs of sources ranged from 7.5 to 30.1. Ratios between all pairs of means ranged from ca. 1 to 20, with only one paired comparison differing significantly (t_{47}=2.09, P=0.042; S6 and N1, Figure 2).

3.3. Effects of infection on colony conditions and growth

Death or supersedure of colonies during four time periods did not associate significantly with average *N. ceranae* infection in months prior to the transition, except for the death of 9 of 102 colonies during September to November ($P>X^2$= 0.02; Table I).

The population of colonies decreased significantly with higher average infection in each of the four time periods (β ranged from −1.4 to –0.3 frames of bees for every million *N. ceranae* per bee; Table II). Despite these significant effects, the proportion of the variation explained by infection was relatively low (R^2=0.05−0.14). Apiary effects on colony population were significant in two of the four time periods (R^2 of 0.16 and 0.29; Table II), with the commercial apiary showing poorer performance of colonies.

4. DISCUSSION

We did not detect differences among genetically diverse honey bee types when we measured average monthly colony infection of *N. ceranae* as an indicator of possible resistance

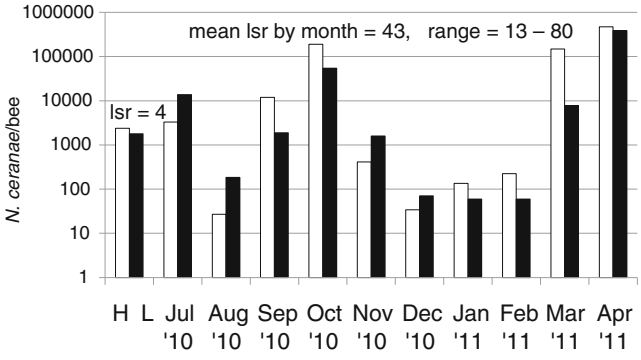

Figure 1. Mean infections of H (*white bars*) and L (*black bars*) colonies across all monthly samples and for every month. Least squares means generated by analysis of variance of $\log_{10}$ transformed values were transformed back to the original scale. The least significant ratio (lsr) required for detection of overall differences between H and L colonies, and mean and range of lsr for differences within months are indicated. None of the observed ratios between overall means or means by month exceeded the required lsr.

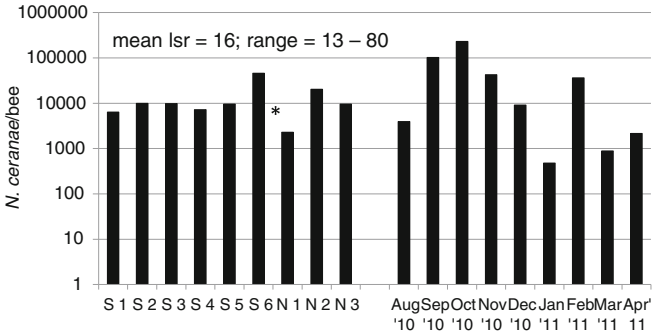

Figure 2. Mean infections of colonies with queens from nine commercial sources across all monthly samples and mean infections of all colonies within each month. A tenth commercial source was omitted from analyses due to queen losses. Least squares means generated by analysis of variance of $\log_{10}$ transformed values were transformed back to the original scale. Mean and ranges of least significant ratios (lsr) required for detection of significant overall differences between pairs of commercial sources are indicated. Only one ratio between means (post-hoc *t*-test) exceeded the respective lsr for the comparison and is indicated by an *

to this parasite. In Experiment 1, considering all months, there were no significant differences between the H and L colonies. Ratios of infection between colonies of the two types only approached the required least significant ratio in one of the 10 months. In Experiment 2, there were no overall differences in infection across different commercial sources, and only in post-hoc tests did the highest (S6) and lowest (N1) commercial sources show a significant difference. The lack of clear differences indicates that selection using field measurements may require refinements in sampling or multigenerational selection on larger populations. Measuring differential infections across patrilines in the same colony (Bourgeois et al. 2012) detected significant differences in Russian colonies. Also, feeding standardized spore dosages to caged workers and comparing survival and infection has shown differences between individual colonies (Malone et al. 1995; Rinderer and Sylvester 1978). Regardless of the ap-

Table I. Average infection [mean±s.e. (number of colonies in each category) as million *N. ceranae* individuals per bee] of monthly colony samples taken within each time period for colonies that were found dead, remained with an original queen or in which the queen was superseded.

Time period	Dead colonies	Live colonies with queen		$P>X^2$	
		Original	Superseded	Death	Supersedure
Jun–Aug	1.1 (1)	1.1±0.1 (102)	1.3±0.8 (5)	0.97	0.77
Sep–Nov	1.7±0.3 (9)	0.9±0.1 (73)	0.6±0.1 (20)	0.02	0.13
Dec–Feb	0.5±0.3 (15)	1.0±0.3 (47)	2.7±2.7 (2)	0.40	0.34
Mar–Apr	2.4 (1)	0.9±0.2 (43)	0.0±0.0 (2)	0.34	0.57

Data are combined from two experiments in two apiaries to assess possible effects of levels of infection on bees. The probability of association between average infection and death (dead vs. live colonies) or supersedure (live with superseded queen vs. live with original queen) was calculated from maximum likelihood estimates of the logistic function and are indicated. Colonies with queens that superseded were not sampled further. The number of live colonies with original queen does not exactly match the sum of sample sizes for means in a subsequent time period: some early deaths or supersedures did not yield samples to contribute to the respective means of colonies in those categories for the time period

Table II. Changes in bee population (in frames of bees) related to levels of *N. ceranae* (in millions of individuals per bee) during four time periods in colonies (n=number of colonies) that remained with original queens in two apiaries.

Time period	n	β	P (β>0)	R^2		
				Combined	*N. ceranae*	Apiary
Jun–Aug	88	−0.835	0.005	0.349**	0.055*	0.286**
Sep–Nov	73	−0.839	0.023	0.218**	0.158**	0.158**
Dec–Feb	51	−0.370	0.050	0.085	0.080*	0.001
Mar–Apr	43	−1.424	0.032	0.136*	0.135*	0.029

The slope (β) of the effect of *N. ceranae* on changes in bee population during each time period and the probability [P (β>0)] of the slope differing from 0 are indicated. R^2 indicates the proportion of total variance explained by either the combined or single contributions of the factors *N. ceranae* and apiary (* or ** indicate when $P>F$ for the factor is below 0.05 or 0.01, respectively)

proach, any selection procedure for resistance would have to produce an economically useful trait that ultimately translates in the reduction of infection in field colonies, the parameter we measured.

The ability to detect differences between stocks was clearly reduced by high variability in colonies of the same origin both at a given time and among times. Given this variability, estimated least significant ratios between genetic types were extremely large and were observed rarely in both experiments. We used large sample sizes (100 bees) because of their greater precision (Bourgeois et al. 2011; F. Eischen, pers. comm.). The distribution of *N. ceranae* in a colony may require improved sampling protocols to better gauge the true level of disease. Infection of individual bees appears to be driven primarily by ingestion of spores, and only small proportions of bees typically develop high infections (Bourgeois et al. 2011, 2012; Smart and Sheppard 2012). The frequency of infected bees, their age, and the actual level of infection can greatly affect the estimates of infection in composite random samples.

Despite this variability in colony-level infection, there were some clear associations between the measured levels of infection and changes in colony condition. There was one significant effect of the average level of infection on the probability of death during the period from September to November. This was the only evident effect out of a total of eight tests that assessed the probability of either death or supersedure among the four time intervals roughly corresponding to summer, autumn, winter and spring. The mortality of 15 colonies from November to February did not reflect a significant association with infection under our relatively mild local winter conditions. Other studies in regions with colder winters also have found no effect of levels of infection on overwintering mortality (Genersch et al. 2010; Guzmán-Novoa et al. 2010; Williams et al. 2010). Fewer colonies (≤5) died or superseded during late summer, winter, and early spring, reducing the possibility for the statistical detection of an association of such events with infection.

Clearer evidence of an association between bee population changes and *N. ceranae* infection was found in colonies that remained alive and with original queens, and this association was apparent in each of the four time periods. Effects of infection were greatest during spring (a decrease of 1.4 frames of bees per million *N. ceranae* in 100 bees during March–April), intermediate in summer and autumn (average decreases of about 0.8 frames of bees per million *N. ceranae*) and least in winter (a decrease of 0.3 frames of bees per million *N. ceranae*; Table II). If this association between infection and bee population is causal, then some common working economic thresholds may

require revisions. Assuming that about one fourth of *N. ceranae* in composite samples are spores, the recommendation of one million spores as an action threshold for treatment would translate into a decrease in colony growth between one and five frames during a 3-month period. Similar negative effects have been found in nearby locations (Eischen et al. 2012) and in Spain (Higes et al. 2008) when colonies treated with fumagillin were compared to untreated colonies over longer time periods than the 10 months of this experiment. Similarly, in groups of untreated colonies, weaker colonies have been found to have the highest levels of spores (Guzman-Novoa et al. 2010) or higher proportions of infected individual bees (Oliver 2012). However, negative effects of *N. ceranae* infection on colony populations are not always evident (Williams et al. 2010; Traver and Fell 2011).

Further research on genetic resistance to *N. ceranae* and on understanding the effects of the parasite on field colonies is needed. Understanding the structure of infection within colonies and possible patterns through time is central to any future work on both topics. Searching for genetic resistance may require initial controlled challenges in cages or screening of larger populations under intense selection pressure. Our finding the lowest levels of infection in colonies from a breeder in the North (N1) who purposefully has used no preventive treatments for either species of *Nosema*, and the highest in an unselected population from the South (S6) suggests that natural selection may be already producing some divergence in expression of resistance.

ACKNOWLEDGMENTS

Lorraine Beaman, Garrett Dodds, David Dodge, Victor Rainey, and Daniel Winfrey assisted with field or laboratory work. Garrett Dodds conducted instrumental inseminations. Debbie Boykin (USDA, ARS) provided statistical advice, and Frank Eischen (USDA, ARS) and Eric Mussen (U. California) provided useful comments on versions of the manuscript. Evergeen Honey Company (Bunkie, LA) provided field colonies and equipment. Partial funding for this project was provided by the Honey Bee Health Improvement Project of the North American Pollinator Protection Campaign.

Preuve négative des effets de l'origine génétique des abeilles sur *Nosema ceranae*, preuve positive des effets de *Nosema ceranae* sur les abeilles

***Apis mellifera* / résistance / nosémose / élevage / sélection**

Negative Belege für Effekte der genetischen Abstammung der Bienen auf *Nosema ceranae*, positive Belege für Effekte von *Nosema ceranae* auf Bienen

***Nosema ceranae* / *Apis mellifera* / Honigbienen / Resistenz / Züchtung / Selektion**

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