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ORIGINAL RESEARCH ARTICLE

Associations among *Nosema* spp. fungi, *Varroa destructor* mites, and chemical treatments in honey bees, *Apis mellifera*

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Nosema spp. and *Varroa destructor* are common parasites of honey bee colonies. Beekeepers routinely treat colonies with the fungicide fumagillin to control *Nosema* and an array of miticides to control *V. destructor*. Interactions between these parasites and chemical treatments are poorly understood. We allocated honey bee colonies to distinct chemical treatment regimes and monitored parasite intensities in the subsequent year. Infections of *Nosema* and infestations of *V. destructor* were positively correlated. Fumagillin was effective at mitigating *Nosema* intensities only over the short term, suggesting that biannual application is essential. *V. destructor* intensities were higher in colonies that had been previously treated with miticides, reasons for this warrant further investigation.

Las asociaciones entre *Nosema* spp. hongos, ácaros *Varroa destructor*, y tratamientos químicos en abejas de la miel, *Apis mellifera*

Nosema spp. y *Varroa destructor* son parásitos comunes de las colonias de abejas de miel. Los apicultores tratan rutinariamente colonias con fumagilina fungicida para controlar *Nosema* y una serie de acaricidas para el control de *V. destructor*. Las interacciones entre estos parásitos y los tratamientos químicos son poco conocidas. Asignamos colonias de abejas de la miel a diferentes regímenes de tratamiento químico y se monitorizaron distintas intensidades de parásitos en el año siguiente. Infecciones de *Nosema* e infestaciones de *V. destructor* se correlacionaron positivamente. La fumagilina fue eficaz para mitigar la intensidad de *Nosema* solamente a corto plazo, lo que sugiere que la aplicación bianual es esencial. Las intensidades de *V. destructor* fueron mayores en las colonias que habían sido tratadas previamente con acaricidas; las razones de este resultado requieren más investigación.

Keywords: beekeeper; coinfection; miticide; fumagillin; *Nosema*; *Varroa destructor*

Introduction

Multi-species parasitic infections are common (Atkinson, Thomas, & Hunter, 2008; Honkavaara, Rantala, & Suhonen, 2009). In some cases, synergistic interactions can occur among parasites when a species acts as a vector (Chen et al., 2004; Le Conte, Ellis, & Ritter, 2010; Yang & Cox-Foster, 2005, 2007) or when parasitism increases host susceptibility to subsequent infections (Bailey, Ball, & Perry, 1983). On the other hand, parasites may have mechanisms for preventing competitors from establishing so that associations between pairs of parasites may be less common than expected by chance (Poulin, 2007; Costa, Tanner, Lodesani, Maistrello, & Neumann, 2011). In the case of economically important parasites, humans have intervened in interspecific parasite associations in a variety of ways, but in particular by application of chemical treatments (pesticides). Consequences of chemical treatments to interactions among parasites have received little attention. Here, we describe field experiments where we manipulated use of

chemical treatments to study interactions between two economically important parasites of honey bees (*Apis mellifera*): *Nosema* spp. fungi and *Varroa destructor* mites.

Honey bees are susceptible to a wide range of parasites that can reduce colony productivity and increase colony mortality (Bailey, 1981; Ball & Allen, 1988; Chen et al., 2004; Cox-Foster et al., 2007; De Jong, Morse, & Eickwort, 1982; Genersch, Evans, & Fries, 2010; vanEngelsdorp & Meixner, 2010). *Nosema apis* fungal infections in honey bee colonies were first recognized a century ago (Fantham & Porter, 1913; Fries et al., 2013; Rennie, White, & Harvey, 1921). However, European honey bees have increasingly become host to the potentially more virulent *Nosema ceranae* that has transferred from its presumed Asian honey bee host, *Apis cerana*, and is now a nearly ubiquitous global presence (Chauzat et al., 2007; Chen, Evans, Smith, & Pettis, 2008; Forsgren & Fries, 2010; Fries, 2010; Fries, Martín, Meana, Garcia-Palencia, & Higes, 2006; Higes, Martín, & Meana, 2006; Klee et al., 2007; Williams, Shafer, Rogers, Shutler, & Stewart, 2008a). Mixed infections of *N. apis*

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and *N. ceranae* may occur in the midgut of adult honey bees (Burgher-MacLellan, Williams, Shutler, Rogers, & Mackenzie, 2010; Chen et al., 2009; Martin-Hernández et al., 2012). *Nosema* spp. spread as cysts in host waste and are associated with increased frequency of colony death and reduced honey production (Bailey, 1955; Fries, 1993, 2010).

Among the most damaging of honey bee parasites is *V. destructor*. *V. destructor* mites typically settle on developing larvae just prior to brood cells being capped, feeding on hemolymph as bees pupate into adults (Yang & Cox-Foster, 2005, 2007). Each female mite that enters a brood cell produces one male and as many as five female mite nymphs, each of which feeds on a developing bee pupa. *V. destructor* are thought to suppress immunity (Le Conte et al., 2010; Yang & Cox-Foster, 2005), although *V. destructor* infestation does not reduce expression of immunity-related genes (Aronstein, Saldivar, Vega, Westmiller, & Douglas, 2012; Kuster, Boncristiani, & Rueppell, 2014; Navajas et al., 2008). *V. destructor* also vectors a number of serious viruses that also are associated with reduced colony health (Bailey, 1981; Ball & Allen, 1988; Chen et al., 2004; Cox-Foster et al., 2007; De Jong et al., 1982; De Miranda et al., 2013; Dietemann et al., 2013; Genersch et al., 2010; vanEngelsdorp & Meixner, 2010).

Arrays of chemical treatments have been developed to counter detrimental effects to honey bees from *Nosema* spp. and *V. destructor* parasites and attendant negative financial impacts on commercial agriculture. Fumagillin, a derivative of *Aspergillus fumigatus*, is the only fungicide available for use in Canada and the USA to fight *Nosema* infections. Although effective at managing nosemosis by either species of *Nosema*, fumagillin does not eradicate these pathogens (Williams, Sampson, Shutler, & Rogers, 2008b; Williams, Shutler, Little, Burgher-MacLellan, & Rogers, 2011). Miticides used to fight *V. destructor* mites include the pyrethroids flumethrin and tau-fluvalinate, the formamidone amitraz, organic acids oxalic and formic, and the organophosphate coumaphos (Boncristiani, Underwood, Schwarz, Evans, & Pettis, 2012; Gregorc, 2005; Gregorc & Smodis Skerl, 2007; Rosenkranz, Aumeier, & Ziegelmann, 2010; Santiago, Otero-Colina, Sánchez, Guzmán, & Vandame, 2000; Wallner & Fries, 2003). Although used to control mite numbers in affected colonies in the short term, miticide resistance is a recurring problem, necessitating the need to switch chemical treatments frequently under a program of Integrated Pest Management (Delaplane, Berry, Skinner, Parkman, & Hood, 2005; Milani, 1999; Rosenkranz et al., 2010). Moreover, chemical treatments may affect health of honey bees (e.g., Christin et al., 2004; Frost, Shutler, & Hillier, 2013; Johnson, Pollock, & Berenbaum, 2009), potentially making them susceptible to other parasites (Gendron et al., 2003; Kiesecker, 2002; Rohr & McCoy, 2010).

Interactions among *Nosema*, *V. destructor*, and chemical treatments have received limited attention.

Moreover, most studies have only examined associations within relatively brief time frames (Alaux et al., 2010; Bermejo & Fernández, 1997; Mariani et al., 2012; Pettis, vanEngelsdorp, Johnson, & Dively, 2012; Wu, Anelli, & Sheppard, 2011). Here, using large numbers of colonies, we tested for associations among *Nosema* spp., *V. destructor*, and chemical treatments used to control them. Examining these issues through large-scale field trials should provide results that are more relevant to real-world situations faced by beekeepers (vanEngelsdorp et al., 2012).

Materials and methods

Twelve beekeepers from across Nova Scotia, Canada participated in this study. Where possible randomly selected colonies were excluded from chemical treatment in fall 2007 and spring 2008 (Table 2). An organic beekeeper provided access to honey bees that were free of *V. destructor* and that received no chemical treatments. Of 280 colonies for which we were able to obtain information, 120 colonies from all beekeepers did not receive fumagillin in fall 2007. With the exception of the organic beekeeper, all other colonies for which records were available were treated with miticide in fall 2007. In spring 2008, colonies were allocated to treated or untreated for fumagillin or miticide primarily by beekeeper. Five beekeepers did not apply fumagillin to all of their colonies and seven beekeepers did not apply miticide to all of their colonies in spring 2008. Two beekeepers agreed to treat only half their colonies with fumagillin and one of these also agreed to treat only half his colonies with miticide. Of 350 colonies, fumagillin was not applied to 178 and miticide was not applied to 187 in spring 2008.

To quantify *Nosema* infections, adult bees were collected from hive entrances in October 2007 (hereafter fall 2007), April 2008 (spring 2008), June and July 2008 (summer 2008), and September 2008 (fall 2008). Approximately 50 bees were collected from entrances of each colony using a battery-operated vacuum modified to divert bees into a small collection jar. Bees were transferred to re-sealable freezer bags and euthanized by freezing at -40°C . For each colony, two replicate suspensions were made, each consisting of 10 ml of distilled water and crushed abdomens of 10 bees (Rogers, Bishop, & Mackenzie, 2002). One micro liter of each suspension was transferred with a loop to a haemocytometer and examined under a phase contrast microscope at 400 magnification and converted to millions of spores per bee (Cantwell, 1970; Rogers et al., 2002). Mean spore counts were then calculated for each colony at each collection time.

To quantify *V. destructor* infestations, adult bees were collected from brood frames in fall 2007, summer 2008, and fall 2008. *V. destructor* counts were not undertaken in spring 2008 because colonies had not yet been removed from winter wrapping. Approximately 200 bees were

Table 1. Beekeepers randomly selected colonies to be excluded from fumagillin and miticide treatments in fall 2007. Chemical treatment records were not available for some of these colonies. Spring 2008 chemical treatments by beekeepers caused further subdivision of chemical treatment regimes (Table 2).

Fumagillin fall 2007	Miticide fall 2007		
	Not treated	Treated	Information not available
Not treated	11	91	18
Treated	0	140	20
Information not available	0	50	20

collected in re-sealable freezer bags from each colony. Bees were euthanized by freezing at -40°C . They were subsequently thawed and washed in winter windshield washer fluid to dislodge mites (British Columbia Ministry of Agriculture & Lands, 2005; Gatien & Currie, 2003). Mites and bees were each counted and numbers of mites per 100 bees were calculated for each colony.

Data were analyzed in SAS 9.3 (Cary, North Carolina, USA). Preliminary analyses revealed significant differences in parasite intensities among beekeepers ($p = .02$ for fall 2007 *V. destructor*, $p < .0001$ for remaining comparisons). Hence, we controlled for beekeeper in analyses of parasite associations. Because beekeepers regularly moved their colonies, often long distances and without notifying us, it was nearly impossible to find and sample each colony at each interval. Hence, sample sizes precluded analyses for combinations of more than one response and one explanatory variable (Table 1). For parasite-parasite associations, we ran general linear mixed models (GLMMs; Proc GLIMMIX) with log(parasite intensity + 1) as response variables and log(parasite intensity + 1) as continuous explanatory variables. We included beekeeper and the interaction with beekeeper as random effects, dropping the interaction term if not

significant to produce final models (Crawley, 2005). Beekeepers varied in enthusiasm for not treating for specific parasites, limiting our ability to control for beekeeper in analyses involving chemical treatments (Table 2). Moreover, beekeepers did not always record information about chemical treatments applied to their colonies (Table 2). Hence, we used general linear models to analyze associations where parasites were response variables and chemical treatments were class explanatory variables. Parametric analyses are typically robust to deviations from normality, particularly with reasonable sample sizes (Winer, Brown, & Michels, 1991), and non-parametric Spearman rank correlations (for parasite-parasite associations) and Kruskal Wallis tests (for parasite-chemical treatment associations) gave similar results, so we report only parametric results.

Results

General

Three hundred and fifty different colonies were sampled for at least two variables. *Nosema* spore counts (*Nosema* intensity) were completed for 156 colonies in fall 2007, 227 colonies in spring 2008, 147 colonies in summer

Table 2. Numbers of colonies subjected to different chemical treatment regimes by beekeepers.

Beekeeper	Chemical treatment regime													Total
	0000	0001	0010	0011	0100	1000	1001	1010	1011	1100	1101	1110	1111	
1												41	10	51
2	11													11
3						30								30
4							10				10			20
5 ^a	18				20									38
6 ^b				18					10				11	39
7											7			7
8 ^c	2					11				12				25
9 ^d		28					10				8			46
10 ^e	5	5	5	5										20
11 ^f			2					11				10		23
12									9				31	40
Total	36	33	7	23	20	41	20	11	19	12	25	51	52	350

Notes: A "0" indicates that chemical treatment was not applied or that that information was not recorded and a "1" indicates that applications occurred. The four-digit regime codes indicate in order whether miticide was applied in fall 2007, fumagillin was applied in fall 2007, miticide was applied in spring 2008, and fumagillin was applied in spring 2008 to a colony. Beekeeper 2 was organic.

^aDid not record whether miticide was applied in fall 2007 for 38 hives.

^bDid not record whether fumagillin was applied in fall 2007 for 18 hives.

^cDid not record whether fumagillin was applied in fall 2007 for 2 hives.

^dDid not record whether fumagillin was applied in fall 2007 for 16 hives.

^eDid not record whether fumagillin was applied in fall 2007 for 20 hives.

^fDid not record whether fumagillin was applied in fall 2007 for 2 hives.

Table 3. Associations among log(parasite intensities + 1)-transformed in honey bee colonies analyzed using general linear mixed models that controlled for beekeeper.

Response variable	Explanatory variable					
	<i>V. destructor</i> fall 2007	<i>Nosema</i> fall 2007	<i>Nosema</i> spring 2008	<i>V. destructor</i> summer 2008	<i>Nosema</i> summer 2008	<i>V. destructor</i> fall 2008
<i>Nosema</i> fall 2007	1,34 +4.3 .05					
<i>Nosema</i> spring 2008	1,25 +1.0 .33	1,119 +1.5 .22				
<i>V. destructor</i> summer 2008		1,32 +<.1 .86	1,86 +3.9 .05			
<i>Nosema</i> summer 2008		1,34 +7.8 .01	1,91 +1 .83	1,116 +<.1 .99		
<i>V. destructor</i> fall 2008	1,12 +3 .58	1,57 +5 .47	1,98 +2.7 .11	1,80 +35.8 <.0001	1,55 -.8 .39	
<i>Nosema</i> fall 2008	1,12 +3.3 .09	1,45 -.9 .34	1,100 -1.5 .23	1,85 +3.1 .08	1,64 +.1 .79	1,133 +.8 .37

Notes: Empty cells below the diagonal occur where there were insufficient data or insufficient variation in data. First line gives degrees of freedom, second line gives F , and third line gives p . Sign in front of F indicates direction of association. Significant results are in bold.

2008, and 169 colonies in fall 2008. We did not determine species of *Nosema* in this study; previous surveys indicate that 90% of infections in our study area are *N. ceranae* (Williams et al., 2008a, 2011). *V. destructor* mite counts (*V. destructor* intensity) were completed for 43 colonies in fall 2007, 162 colonies in summer 2008, and 162 colonies in fall 2008.

Parasite–parasite associations

In GLMMs controlling for beekeeper, 16 of 19 parasite–parasite associations were positive (two-tailed binomial

test; $p = .003$) and four were significantly so (Table 3). However, this is partly because each beekeeper subjected their bees to a different chemical treatment regime (Table 2). In fall 2007, *Nosema* and *V. destructor* intensities were significantly and positively correlated. Summer 2008 *V. destructor* intensities were significantly and positively associated with *Nosema* spring 2008 intensities (Figure 1). *Nosema* intensities in spring 2008 were positively associated with *V. destructor* intensities in summer 2008. Only two beekeepers in this analysis had more than five colonies sampled, and *Nosema*

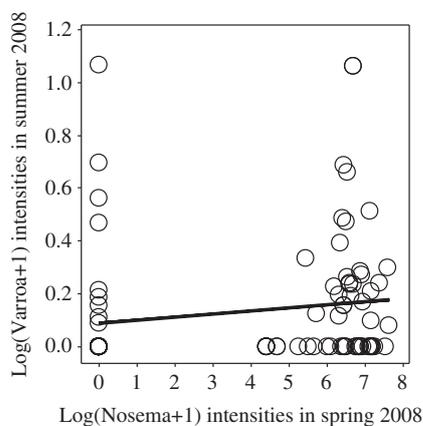


Figure 1. Summer 2008 *Varroa destructor* intensities were higher in colonies that had higher spring 2008 *Nosema* intensities ($F_{1,86} = 3.9$, $p = .05$). This association was not significant if zero-intensity *Nosema* colonies were excluded ($F_{1,50} = .6$, $p = .45$).

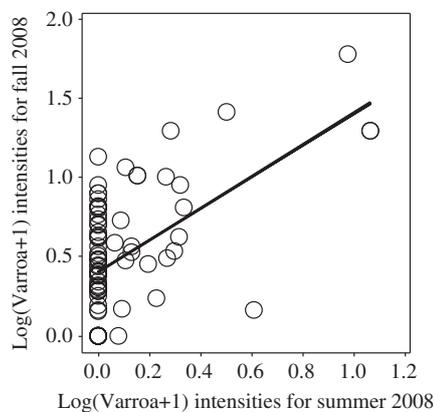


Figure 2. Fall 2008 *Varroa destructor* intensities were higher in colonies that had higher summer 2008 *V. destructor* intensities ($F_{1,80} = 35.8$, $p < .0001$). This association remained significant if zero-intensities from summer 2008 were excluded ($F_{1,17} = 7.5$, $p = .01$).

Table 4. Associations among parasite intensities (*V. destructor* counts/100 bees and log(*Nosema* counts + 1) and chemical treatment in honey bee colonies from fall 2007 to fall 2008 analyzed using general linear models.

Response variable	Explanatory variable		
	Fumagillin fall 2007	Miticide spring 2008	Fumagillin spring 2008
<i>Nosema</i> spring 2008	1,205 -3.5 .06		
<i>V. destructor</i> summer 2008	1,110 -.4 .53	1,160 +23.2 <.0001	1,160 -.2 .68
<i>Nosema</i> summer 2008	1,114 -9.0 .003	1,145 -1.2 .27	1,145 -8.5 .004
<i>V. destructor</i> fall 2008	1,126 +10.0 .002	1,160 +55.2 <.0001	1,160 +9.5 .003
<i>Nosema</i> fall 2008	1,116 +.5 .48	1,167 -<.1 .96	1,167 +1.3 .26

Notes: Empty cells below the diagonal occur where there were insufficient data or insufficient variation in data. First line gives degrees of freedom, second line gives *F*, and third line gives *p*. A positive sign in front of *F* indicates that chemical treatment is associated with increased parasite intensities. Significant results are in bold.

associations within these beekeepers were not significant ($ps \geq .38$). Finally, there was a significant association between *V. destructor* intensities in summer and fall 2008 (Figure 2).

Parasite-chemical treatment associations

Of 13 parasite-chemical treatment associations, 7 were negative (two-tailed binomial test; $p = .78$). Two associations were significantly negative and four were significantly positive (Table 4). Higher *V. destructor* intensities were observed in summer and fall 2008 for colonies treated with miticides in spring 2008 than for untreated colonies. Lower *Nosema* intensities in summer 2008 were observed following fumagillin treatments in fall 2007 and spring 2008. Lower *Nosema* intensities in spring 2008 were also observed following fall 2007 fumagillin treatment, although results were of borderline significance. Higher *V. destructor* intensities in fall 2008 were also observed following fumagillin treatments in fall 2007 and spring 2008.

Discussion

As is typical of parasite data, significant variation in intensities was observed; thus our results must be interpreted cautiously. Parasite intensities fluctuated seasonally with highest *Nosema* spore counts occurring in summer 2008 and highest *V. destructor* mite counts in fall 2008. As expected, colonies that had *Nosema* infections in one season tended to have higher intensity infections in subsequent seasons and colonies that had *V. destructor* infestations in one season tended to have higher intensity infections in subsequent seasons, suggesting possible lag effects on honey bee health following infection.

Colonies that had *V. destructor* infestations were more likely to have higher intensity *Nosema* infections and colonies with *Nosema* infections were more likely to have higher intensity *V. destructor* infestations. These relationships persisted over subsequent seasons. This is consistent with Bermejo and Fernández (1997), Le Conte et al. (2010), Mariani et al. (2012), and Higes, Meana, Bartolomé, Botías, and Martín-Hernández (2013) who suggested a synergistic relationship between *Nosema* spp. and *V. destructor* mites. Hypotheses for these synergisms include increased permeability of peritrophic membranes following *V. destructor* infestation that may increase susceptibility to *Nosema* infection, decreased fat body stores following *Nosema* infection that may increase susceptibility to *V. destructor* infestation, and behavioral changes due to increased energetic stress following *Nosema* infection that may increase susceptibility to *V. destructor* infestation. Both *V. destructor* and *N. ceranae* have been implicated in immunosuppression and decreased vitellogenin expression in honey bees (Antúnez et al., 2009; Yang & Cox-Foster, 2007), possible stressors that could also facilitate co-infection.

Although beekeepers changed miticide regimes at each treatment, colonies that received miticide treatment in spring 2008 had higher *V. destructor* counts in summer and fall 2008 than untreated colonies. This is consistent with beekeepers' fears that *V. destructor* mites have developed at least partial resistance to the array of commercially available miticides. The common practice of treating colonies with combinations of miticides could also have led to inadvertent drug interactions that increase toxicity to *A. mellifera* and further compromised honey bee health (Johnson, Dahlgren, Siegfried, & Ellis, 2013). Although this study could not confirm a causal relationship between miticide use and increased

V. destructor counts, non-chemical integrated pest management may be a more economical and effective alternative (Delaplane et al., 2005). Colonies treated with fumagillin in fall 2007 or spring 2008 also had higher *V. destructor* counts in fall 2008 than untreated colonies. This may be attributable to changes in honey bee brood rearing behavior in colonies treated with fumagillin (Webster, 1994).

Consistent with previous research (Williams et al., 2008a, 2011), *Nosema* counts in spring and summer 2008 were lower in colonies that had been treated with fumagillin in fall 2007 or spring 2008 than those that were not treated. By fall 2008, beneficial effects of fumagillin had faded, suggesting that repeated application biannually is critical to controlling intensity of *Nosema* infestations. Laboratory research by Huang, Solter, Yau, and Imai (2013) suggested that fumagillin degradation contributed to preferential proliferation by *N. ceranae* and that even biannual fumagillin application may not be sufficient to suppress infection over the long term. Further research is required to determine possible causes, including potential increased degradation of fumagillin by high late summer temperatures or reduced effective dosage per bee due to larger colony sizes in summer months. No significant relationship was observed between *Nosema* intensity and application of miticides.

These results demonstrate that beekeepers' efforts to mitigate infections by each parasite have effects against the other parasite and that these beneficial effects can last over several seasons. Essentially, controlling *V. destructor* infestations in colonies may be vital to mitigating *Nosema* infection, and managing *Nosema* infections may be beneficial to controlling spread of *V. destructor* mites. Colonies in this study were treated with a variety of miticides commercially available in Canada. *V. destructor* infestations were more intense regardless of the specific miticides used, suggesting that resistance develops quickly in mites. Additionally, Botías et al. (2012) suggested that behavioral changes in honey bees infected by *Nosema* reduced efficacy of some miticides used to treat *V. destructor*. These same miticides can shorten lifespans, compromise immunocompetence, and affect metabolic processes of honey bees (Boncristiani et al., 2012). Fumagillin, while effective in the short-term to control *Nosema* infections, was associated with more intense *Nosema* infections in the long-term and more intense *V. destructor* infestations. Non-chemical parasite control options may be a more viable, albeit more labor-intensive, long-term solution.

Our research has revealed a number of potential interactions among *Nosema* spp., *V. destructor* mites, and chemical treatments. Although we found significant correlations among these variables, causality may be due to other factors, including differences in management styles among beekeepers. These associations merit further scrutiny both for economic and scientific reasons; mechanisms that appear to produce these associations may be very instructive to managing honey bee colonies.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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