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Original article

The microsporidian *Nosema ceranae*, the antibiotic Fumagilin-B®, and western honey bee (*Apis mellifera*) colony strength*

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Abstract – Western honey bees (*Apis mellifera*) are under threat from a number of emerging pathogens, including the microsporidian *Nosema ceranae* historically of Asian honey bees (*Apis cerana*). Because of its recent detection, very little is known about the biology, pathology, and control of *N. ceranae* in western honey bees. Here we investigated effects of the antibiotic Fumagilin-B®, which is commonly used to control the historical *Nosema* parasite of western honey bees *Nosema apis*, on *N. ceranae* and effects of *N. ceranae* on colony strength (i.e., number of bees and amount of capped and uncapped brood, honey, and pollen) and colony mortality. Similar to our previous study, fall Fumagilin-B® treatment lowered, albeit weakly, *N. ceranae* intensity the following spring. However, *N. ceranae* was not associated with variation in colony strength measures or with higher colony winter mortality.

***Apis mellifera* / honey bee / *Nosema ceranae* / fumagillin / pathology**

1. INTRODUCTION

In recent years, the apiculture industry has been besieged by a spate of emerging diseases. The microsporidian *Nosema ceranae* parasitizes both the Asian (*Apis cerana*) and western (*Apis mellifera*) honey bee, although it is more closely related to *Nosema bombi* that parasitizes bumble bees than it is to *Nosema apis* that parasitizes western honey bees (Shafer et al., 2009). *N. ceranae* jumped from the Asian to the western honey bee some time before 1998 (Paxton et al., 2007; Chen et al., 2008). It is now ubiquitous in western honey bees (e.g. Higes et al., 2006; Klee et al., 2007; Chen et al., 2008; Williams et al., 2008a), and has displaced the historical *N. apis* parasite of western honey bees in many regions (Klee et al., 2007; Chen et al., 2008; Williams et al., 2008b).

Very little is known about *N. ceranae* biology and pathology in western honey bees (Fries, 2009). Like *N. apis*, it primarily infects midgut epithelia; however, *N. ceranae* appears to be more pathogenic when inoculated experimentally (Higes et al., 2007; Paxton et al., 2007). It is possible that unique strains of *N. ceranae* (Williams et al., 2008a) vary in virulence, so there may be geographic differences in effects on beekeeping industries. In Spain, *N. ceranae* maintains year-round

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high prevalence (proportion of bees infected) (Martín-Hernández et al., 2007), in contrast to the early summer peaks of infection that characterize *N. apis* (Pickard and El-Shemy, 1989). Moreover, *N. ceranae* can cause sudden collapse of seemingly asymptomatic colonies (Higes et al., 2008). In the United States, the cause of Colony Collapse Disorder, a phenomenon that occurs in colonies when adult bees rapidly disappear from a colony (i.e., absence of dead bees in the colony) lacking damaging levels of *V. destructor* and *Nosema* populations, leaving behind unattended brood and intact food stores (vanEngelsdorp et al., 2009), remains unexplained. It is likely that multiple stressors acting alone or in concert with each other weaken bees and allow for opportunistic pathogens to invade and kill the colony (vanEngelsdorp et al., 2009). In Canada, large-scale unexplained colony collapses have not been observed, despite above-average colony losses in recent years. Suspected contributors include weather, the parasitic mite *Varroa destructor*, and *N. ceranae*, but few supporting data exist. Regardless of these differences, data on pathology of *N. ceranae*, and efficacy of control measures used against it, are of extreme interest.

Numerous control methods exist for *N. apis* in western honey bees, including heat treatment (Cantwell and Shimanuki, 1969), fumigation (Bailey, 1957), and replacement of aged equipment (Fries, 1988). Most common in Canada and the United States is oral administration of the antibiotic fumagillin dicyclohexylammonium (hereafter, fumagillin) with sugar syrup during fall feeding (Gochnauer and Furgala, 1969), but an additional spring application is often recommended for severe infections. With the exception of Spain, fumagillin is no longer registered for use in Europe because honey cannot contain antibiotic residues on that continent (Europa, 2009). Medicated colonies have lower *N. apis* intensities the following spring, although no differences in survival were observed with different fumagillin concentrations (Szabo and Heikel, 1987). It is not known what control methods used against *N. apis* in western honey bees are appropriate for *N. ceranae*, especially because fumagillin was ineffective against closely-related *N. bombi* in the bumble bee *Bombus occidentalis* (Whittington and Winston, 2003). In eastern Canada, fumagillin applied at the manufacturer's recommended fall dosage for *N. apis* (administered as Fumagilin-B®; one treatment of 190 mg fumagillin per colony) controlled *N. ceranae* over the wintering period (Williams et al., 2008b); however, we did not account for potential variation among beekeeping operations that differ, for example, in location of wintered colonies, in condition of colonies in fall, or in use of additional parasite management treatments. In Spain, fumagillin (administered as Fumadil-B®; four weekly treatments for a total of 120 mg fumagillin per colony) also controlled *N. ceranae* within a 1-yr period, and significantly reduced the risk of depopulation (Higes et al., 2008).

Using colonies over-wintered in a single apiary, we investigated effects of Fumagilin-B® on *N. ceranae* and effects of *N. ceranae* on western honey bee colony strength (i.e., size of adult worker population, and amounts of capped and uncapped brood, honey, and pollen) and colony mortality. In parallel, we repeated our earlier study (Williams et al., 2008b) using colonies over-wintered by seven different beekeepers.

2. MATERIALS AND METHODS

Two experiments were conducted between August 2007 and May 2008 in Nova Scotia, Canada.

2.1. Experiment 1

We collected worker honey bees from hive entrances of 57 two-brood-chambered commercial colonies in three apiaries in Hants County on 4 September 2007 using a portable vacuum device. Bees were kept at −20 °C until spore suspensions could be made for each colony by adding 30 mL of distilled water to crushed abdomens of 30 randomly-selected individuals. We estimated *Nosema* intensity per colony using light microscopy and a haemocytometer. For each sample we counted the number of spores in 80 haemocytometer squares (i.e., 5 groups of 16 squares) (Cantwell, 1970; Rogers and Williams, 2007).

Between 6 and 12 September 2007, we visually estimated (in number of frames out of 10) coverage with bees, capped and uncapped brood, honey, and pollen (we refer to these as colony strength measures) for every other frame (due to time constraints) in the upper and lower brood boxes of each colony. Presence of eggs and disease, such as American and European foulbroods, chalkbrood, and deformed-winged workers, were also recorded.

Colonies were moved to a single winter apiary at the end of September 2007. Based on colony assessments, 47 queenright colonies were chosen for the experiment on 10 October 2007; 26 colonies received 8 L each of fumagillin-medicated 2:1 (determined by weight) sugar syrup (one treatment of 190 mg fumagillin per colony) and 21 colonies received 8 L of un-medicated 2:1 sugar syrup. Colonies were placed on 4-way commercial beekeeping pallets grouped as medicated or un-medicated colonies to minimize bees visiting colonies of the opposing treatment group. All colonies received equal treatments of Check-Mite® in mid-September 2007 and Terramycin® in mid-October 2007 to control *V. destructor* and American foulbrood, respectively. In addition, sticky boards inserted on 11 October 2007 were used to monitor 24-hour natural drop of *V. destructor*.

Bees were re-sampled from hive entrances for *Nosema* quantification (see above) on 21 April 2008 and for colony strength between 28 April and 6 May 2008. Presence of eggs and disease (see above) were recorded, and sticky boards were inserted on 27 April 2008 to monitor *V. destructor*. Colony mortality was determined by the beekeeper; dead colonies were defined as those without a queen or those with <2 frames of bees during spring 2008 inspections.

All statistical analyses were performed using R. 2.7.2., unless otherwise stated. Repeated-measures ANOVAs were used to compare hive strength variables, *Nosema* intensity, and *V. destructor* intensity between control and fumagillin-treated groups. A discriminant function analysis was used to compare a composite of the colony strength variables using SAS 9.2. Where appropriate, data were square-root transformed to improve fit to normality. Perfect fit could not always be achieved; nonetheless, our analyses are likely to be robust because of our large sample sizes. We used logistic regression to analyse the relationship between fall 2007 *Nosema* spore intensity and winter mortality. We tested for differences in colony mortality between treatment groups using χ^2 tests.

2.2. Experiment 2

Worker honey bees were collected from the hive entrances of 130 commercial colonies from 7 different beekeeping operations in Kings, Hants, Colchester, Cumberland, and Guysborough counties between 20 and 26 August 2007 using a portable vacuum device. Number of colonies sampled per operation ranged between 8 and 37. Bees were kept at −20 °C until *Nosema* could be assessed (see above). Between mid-September 2007 and early October 2007, approximately half of the colonies sampled in each apiary received 8 L of fumagillin-medicated 2:1 sugar syrup (one treatment of 190 mg fumagillin per colony), depending on beekeeping operation; remaining colonies received 8 L of un-medicated 2:1 sugar syrup. All colonies were managed for *V. destructor* and American foulbrood by their respective beekeepers. Worker bees were re-sampled for *Nosema* quantification from hive entrances of surviving (defined above) colonies between 9 and 16 April 2008.

As before, *Nosema* data were square-root transformed, and repeated measures and split-plot ANOVAs were used to analyse effects of beekeeper and fumagillin treatment on *N. ceranae*. We used logistic regression to analyse the relationship between fall 2007 *N. ceranae* and winter mortality, and χ^2 tests to analyse differences in colony mortality between treatment groups.

2.3. *Nosema* species identification

Molecular analyses were performed using duplex PCR (see Williams et al., 2008b) and primers 321APIS-FOR and 321APIS-REV for *N. apis* and 218MITOC-FOR and 218MITOC-REV for *N. ceranae* (Martín-Hernández et al., 2007) on all Experiment 1 colony suspensions created in spring 2008 that contained *Nosema* spores, in addition to a few without spores. Analyses were not performed on Experiment 2 samples because a previous survey of the same beekeepers identified *N. ceranae* in >90% of colonies tested (Williams et al., 2008b).

3. RESULTS

3.1. Experiment 1

Nosema ceranae was not significantly lower in fumagillin-medicated than in control

Table I. Summary statistics of *Nosema* spore counts (in millions) for control and fumagillin-treated honey bee colonies from seven beekeepers in Nova Scotia during fall 2007 and spring 2008, and corresponding repeated measures ANOVA results (significant differences in bold).

Beekeeper	Fall 2007				Spring 2008				df	*F*	*P*
	Control		Fumagillin-treated		Control		Fumagillin-treated				
	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
1	0.3	0.5	0.9	1.2	0.1	0.3	<0.1	0.1	1.24	0.3	0.58
2	2.5	0.2	3.2	1.0	5.4	11.2	1.3	2.0	1.14	0.2	0.64
3	2.2	2.4	2.1	1.3	5.8	5.4	3.2	2.3	1.15	0.8	0.40
4	2.7	1.6	1.3	0.9	5.5	2.0	0.9	0.9	1.8	24.6	**<0.01**
5	3.5	2.7	3.3	2.1	8.3	7.7	6.4	10.0	1.12	0.1	0.78
6	1.5	1.4	0.7	0.7	5.9	4.8	0.1	0.2	1.5	13.1	**0.02**
7	1.6	1.2	1.9	2.3	0.5	1.2	<0.1	<0.1	1.13	0.7	0.43

colonies that survived to spring 2008 (control colonies' mean ± SD in millions for 2007, 2008: 1.3 ± 1.5, 2.2 ± 5.4, fumagillin-treated colonies' mean ± SD in millions for 2007, 2008: 0.9 ± 1.1, 0.3 ± 0.7, repeated measures ANOVA: $F_{1,37} = 3.5$, $P = 0.07$). There were no significant differences between treatment groups for estimates of bees ($F_{1,37} = 0.1$, $P = 0.76$), capped ($F_{1,37} = 0.8$, $P = 0.37$) and uncapped ($F_{1,37} = 1.16$, $P = 0.29$) brood, honey ($F_{1,37} = 1.9$, $P = 0.17$), or pollen ($F_{1,37} = 0.2$, $P = 0.68$) (Figs. 1a–e). Moreover, discriminant function analyses of these variables failed to distinguish between control and fumagillin-treated colonies in either 2007 (32/47 colonies correctly discriminated, $F_{5,41} = 1.5$, $P = 0.23$) or 2008 (27/40 colonies correctly discriminated, $F_{5,34} = 0.9$, $P = 0.48$), and there were no significant differences for *V. destructor* (control colonies' mean ± SD for 2007, 2008: 21.9 ± 23.6, 0.4 ± 0.6, fumagillin-treated colonies' mean ± SD: 17.3 ± 17.1, 0.4 ± 0.6, repeated measures ANOVA: $F_{1,35} = 0.3$, $P = 0.60$). Fall 2007 *Nosema* spore counts and winter mortality were not related when all colonies (logistic regression: $Z = 0.6$, $P = 0.52$), control colonies ($Z = 0.3$, $P = 0.71$), or fumagillin-treated colonies ($Z = 0.6$, $P = 0.58$) were grouped together. Similarly, there was no relationship between fumagillin treatment and winter mortality ($\chi^2_1 < 0.1$, $P = 0.93$), with 3/21 control and 4/26 fumagillin-treated colonies dying.

Additional diseases were infrequent; one fumagillin-treated colony contained 67 and 30 cells of chalkbrood mummies in fall and spring, respectively, and two fumagillin-treated colonies contained workers with deformed wings. American and European foulbroods were not observed.

3.2. Experiment 2

Nosema was significantly lower in fumagillin-medicated colonies (repeated measures ANOVA: $F_{1,103} = 4.0$, $P = 0.05$) and remained so after we controlled for beekeeper (split-plot ANOVA for beekeeper and treatment: $F_{1,97} = 5.0$, $P = 0.03$); however, *Nosema* was only significantly lower in treated colonies for 2/7 beekeepers (Repeated measures ANOVAs; Tab. I). Similar to Experiment 1 results, there were no relationships between fall 2007 *Nosema* spore counts and winter mortality when all colonies (logistic regression: $Z = 0.4$, $P = 0.66$), control colonies ($Z = 2.5$, $P = 0.21$), or treated colonies ($Z = -0.6$, $P = 0.53$) were grouped together. Similarly, there was no relationship between fumagillin treatment and winter mortality ($\chi^2_1 = 0.3$, $P = 0.56$), with 11/53 control and 14/52 fumagillin-treated colonies dying.

3.3. *Nosema* species identification

Fifteen of 20 samples molecularly analyzed had *N. ceranae*, and one had both *N. ceranae* and *N. apis*. The remaining 4 samples failed to amplify any PCR product, possibly due to DNA degradation.

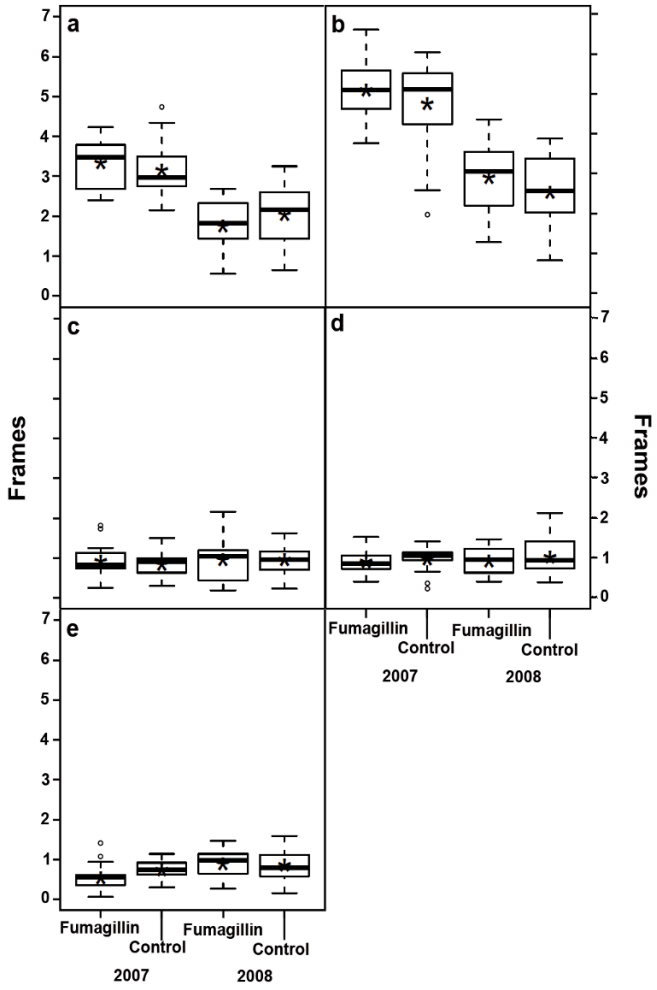

Figure 1. Comparisons between control and fumagillin-treated honey bee colonies in fall 2007 and spring 2008 for number of frames out of 10 of: (a) adult bees; (b) honey; (c) pollen; (d) capped brood; and (e) uncapped brood. Boxplots show interquartile range (box), median (black line within interquartile range), data range (dashed vertical lines), and outliers (open dots). Asterisks represent means. Estimates were made for every other frame in each colony.

4. DISCUSSION

Overall, Fumagilin-B® reduced *N. ceranae* intensities the following spring, but results were highly variable both within and among beekeepers, and the degree of control was weak (i.e, $P = 0.07$ and 0.03 for experiments 1 and 2, respectively). Among a large number of potential explanations for this variability, a colony's initial strength and food intake (i.e., sugar syrup and thus dosages of fumagillin) in fall may partially explain differences in effectiveness of fumagillin. Similarly, variability could arise from variation in stress from previous summer commercial pollination activities, forage availability, and winter weather, as well as beekeeper management; however, very little is known about factors influencing *N. ceranae* growth and development. Our data highlight the importance of large sample sizes and

of monitoring conditions under which bees are maintained in evaluating *N. ceranae* and its management in commercial colonies.

Despite lower *N. ceranae* intensity in fumagillin-treated colonies, there were no differences in indices of colony strength or winter mortality between treatment groups the following spring, suggesting that both fumagillin and *N. ceranae* had little effect on colonies under these experimental conditions. There are a number of possible explanations for this lack of pathology.

First, haplotypes of *N. ceranae* may differ in virulence, and the *N. ceranae* haplotype in Nova Scotia, as well as other regions of Canada and the United States (Williams et al., 2008a), may be less virulent than those found in other regions of the world, such as in Spain where it is implicated in colony die-offs (Higes et al., 2008).

Second, damaging thresholds for *N. ceranae* have not been concretely quantified, and only limited data set the threshold at >1 000 000 *N. apis* spores per bee. *N. ceranae* damage thresholds may not have been met in this study, although in fall 2007 we observed mean spore loads of >1 000 000 in 6 of 7 beekeepers' colonies. More research is needed to understand damage thresholds for both *N. apis* and *N. ceranae* under a number of environmental conditions, and for a diversity of haplotypes, because Nova Scotia colonies appear to be unaffected in spring when mean spore counts of foraging bees were >10 million the previous fall (Williams et al., 2010).

Third, weather during our experiments was normal, with beekeepers reporting plentiful pollen supplies and nectar flow during late summer (J. Moran, unpubl. data); therefore, colonies may have been healthy and not susceptible to *N. ceranae*.

Fourth, the experiment was terminated after spring assessments because beekeepers were moving their colonies to pollinate crops. Effects of *N. ceranae* could have occurred after we stopped collecting data; colonies may have undergone a "false recovery" as reported by Higes et al. (2008). We consider this unlikely because experimental colonies were acquired from a commercial operation that had not treated colonies with fumagillin the previous fall; therefore, colonies would not have had low spore counts in spring 2007 characteristic of the "asymptomatic" phase (Higes et al., 2008).

Fifth, it is possible that proportion of infected foragers, rather than spore counts determined from composite samples of 30 foraging bees, are a better indicator of colony disease from *N. ceranae* infection (Higes et al., 2008). This was also true for *N. apis* and honey yield, although both individual and composite estimates were strongly correlated with each other (Fries et al., 1984).

Lastly, because *N. ceranae* intensity did not differ significantly between controls and fumagillin-treated colonies in Experiment 1, we might not expect to have differences in our indices of colony strength.

Very little is known about consequences of *N. ceranae* to western honey bees because of its recent detection (Huang et al., 2007). Understanding factors influencing colony-level pathology of *N. ceranae* is crucial, particularly due to conflicting data suggesting that the parasite is more pathogenic in Europe than in North America (Higes et al., 2008; vanEngelsdorp et al., 2009). Although fumagillin applied at manufacturer-recommended rates appears to control *N. ceranae* to a certain extent, more work is needed to determine ideal dosages of fumagillin for *N. ceranae*-infected colonies during the season, such as amount of active ingredient required and number of applications per treatment. Recent attention has focussed on how multiple factors affect colony health, including parasites and pesticides (vanEngelsdorp et al., 2009). Until influences of all important stressors, both singly and in combination, are known, it may be difficult to understand differences in strength and mortality among western honey bee colonies.

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La microsporidie *Nosema ceranae*, l'antibiotique Fumagillin-B® et le bon état des colonies d'abeilles (*Apis mellifera*).

***Apis mellifera* / *Nosema ceranae* / antibiotique / pathologie / état sanitaire**

Zusammenfassung – Der Einfluss des Mikrosporidiums *Nosema ceranae* und des Antibiotikums Fumagilin-B® auf die Volksstärke der Honigbiene *Apis mellifera*. Die westliche Honigbiene *Apis mellifera* wird von einer ganzen Reihe neuer Pathogene bedroht, darunter auch dem Mikrospridium *Nosema ceranae*, ursprünglich ein Parasit der östlichen Honigbiene *Apis cerana*. Da *N. ceranae* erst kürzlich entdeckt wurde, ist bisher erst wenig über Biologie, Pathologie und Bekämpfung dieses Parasiten bei *A. mellifera* bekannt (Fries, 2009). In Spanien kann der Nosema-Befall zu einem plötzlichen Zusammenbruch bei anscheinend symptomlosen Bienenvölkern führen (Higes et al., 2008), während der Erreger in Nordamerika offensichtlich deutlich weniger pathogen ist (vanEngelsdorp et al., 2009). Es gibt zahlreiche Bekämpfungsmethoden gegen *N. apis*, wovon in Kanada und den USA die orale Applikation mit dem Antibiotikum Fumagillin-Dicyclohexylammonium (=Fumagillin) in Zuckerwasser die am weitesten verbreitete Methode ist.
Wir untersuchten den Effekt von Fumagilin-B® auf *N. ceranae* und auf die Volksstärke von *A. mellifera* (adulte Bienenpopulation, Umfang der offenen und der verdeckelten Brut sowie von Honig und Pollen) sowie den Effekt von *N. ceranae* auf die Mortalität der Bienenvölker. Wir verwendeten dabei Versuchs-Bienenvölker, die zuvor am selben Bienenstand überwinterten. Parallel dazu wiederholten wir unsere früheren Untersuchungen (siehe Williams et al., 2008b) mit Bienenvölkern, die von 7 verschiedenen Imkern überwintert wurden, um die Variation durch die imkerlichen Betriebsweisen mit zu berücksichtigen.
N. ceranae hatte weder einen Einfluss auf die verschiedenen Parameter der Volksstärke noch auf die Überwinterungsmortalität der Bienenvölker. Ähnlich wie in unserer vorangegangenen Untersuchung reduzierte eine Behandlung mit Fumagilin-B® im Herbst allgemein den *N. ceranae*-Befall im folgenden Frühling, allerdings mit erheblichen Schwankungen sowohl innerhalb der Bienenstände als auch zwischen den Imkern. Dies könnte auf mehrere Faktoren zurückzuführen sein, darunter Stress der Bienenvölker durch Bestäubungsaktivitäten im vorangegangenen Sommer, Nahrungsverfügbarkeit, Wetter und imkerliches Management.
Das Verständnis der Pathologie von *N. ceranae* auf der Ebene des Bienenvolkes ist von größter Bedeutung, insbesondere angesichts widersprüchlicher Daten, nach denen der Parasit in Europa sehr viel gefährlicher sein soll als in Nordamerika (Higes et al., 2008; vanEngelsdorp et al., 2009). Die deutlich zunehmenden globalen Bienenvolkverluste in letzter Zeit haben vermutlich multifaktorielle Ursachen. Solange wir nicht den Einfluss aller wichtigen Stressoren – allein und in Kombination – kennen, wird es schwierig sein, die Unterschiede in Volksstärke und Mortalität bei Bienenvölkern der westlichen Honigbiene zu verstehen.

***Apis mellifera* / Honigbiene / *Nosema ceranae* / Fumagillin / Pathologie**

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