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INVERTEBRATE MICROBIOLOGY

Rapid Gastrointestinal Passage May Protect *Bombus terrestris* from Becoming a True Host for *Nosema ceranae*

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ABSTRACT Pollination provided by managed honey bees as well as by all the wild bee species is a crucial ecosystem service contributing to the conservation of biodiversity and human food security. Therefore, it is not only the health status of honey bees but also the health status of wild bees that concerns us all. In this context, recent field studies suggesting interspecies transmission of the microsporidium parasite *Nosema ceranae* from honey bees (*Apis mellifera*) to bumblebees (*Bombus* spp.) were alarming. On the basis of these studies, *N. ceranae* was identified as an emerging infectious agent (EIA) of bumblebees, although knowledge of its impact on its new host was still elusive. In order to investigate the infectivity, virulence, and pathogenesis of *N. ceranae* infections in bumblebees, we performed controlled laboratory exposure bioassays with *Bombus terrestris* by orally inoculating the bees with infectious *N. ceranae* spores. We comprehensively analyzed the infection status of the bees via microscopic analysis of squash preparations, PCR-based detection of *N. ceranae* DNA, histology of Giemsa-stained tissue sections, and species-specific fluorescence *in situ* hybridization. We did not find any evidence for a true infection of bumblebees by *N. ceranae*. Through a series of experiments, we ruled out the possibility that spore infectivity, spore dosage, incubation time, or age and source of the bumblebees caused these negative results. Instead, our results clearly demonstrate that no infection and production of new spores took place in bumblebees after they ingested *N. ceranae* spores in our experiments. Thus, our results question the classification of *N. ceranae* as an emerging infectious agent for bumblebees.

IMPORTANCE Emerging infectious diseases (EIDs) pose a major health threat to both humans and animals. EIDs include, for instance, those that have spread into hitherto naive populations. Recently, the honey bee-specific microsporidium *Nosema ceranae* has been detected by molecular methods in field samples of bumblebees. This detection of *N. ceranae* DNA in bumblebees led to the assumption that *N. ceranae* infections represent an EID of bumblebees and resulted in speculations on the role of this pathogen in driving bumblebee declines. In order to address the issue of whether *N. ceranae* is an emerging infectious agent for bumblebees, we experimentally analyzed host susceptibility and pathogen reproduction in this new host-pathogen interaction. Surprisingly, we did not find any evidence for a true infection of *Bombus terrestris* by *N. ceranae*, questioning the classification of *N. ceranae* infections as EIDs of bumblebees and demonstrating that detection of microsporidian DNA does not equal detection of microsporidian infection.

KEYWORDS bumblebee, *Bombus terrestris*, microsporidia, *Nosema ceranae*, emerging infectious disease, experimental infection

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Animal pollination is an important ecosystem service to agriculture because about 70% of the leading crops directly used for human food increase production with animal pollination (1). Among the pollinating animals, wild-insect pollinators play a major role. A recent study analyzed 41 crop systems worldwide and found positive associations of fruit set with flower visitation by wild insects in all of those systems (2). In contrast, flower visitation by managed honey bees (*Apis mellifera*) was related to a significant increase in fruit set in only 14% of the surveyed systems (2). Moreover, pollination by wild insects was more effective than pollination by honey bees (2), further emphasizing the indispensable role of wild-insect pollinators in human food security. In addition, it was shown that pollination by managed honey bees supplemented (rather than substituted for) pollination by wild insects and that the activities of *Apis* and non-*Apis* bees even have synergistic effects for pollination (2–4). Thus, although about 90% of the commercial pollination in agriculture is provided by managed honey bees, because they can be used where and when they are needed (5), human food production heavily depends also on wild-insect pollination.

The role of honey bees as indispensable managed pollinators in agriculture (5–7) together with recent reports on severe honey bee colony losses (8–10) led to increased interest and research in all fields of honey bee health in the past 15 years. In the wake of this development and with the increasing awareness of the importance of other insects such as wild bees as pollinators, the health of bees in general (honey bees and wild bee species) came into focus. The picture that emerged from those studies is that flower-visiting insects, cooccupying the same ecological niche, share many pathogens because not only intraspecies transmission but also interspecies transmission occurs between different species, with flowers serving as pathogen hubs (11–17).

Honey bees known to carry a plethora of different pathogens were suspected to be the source for interspecies transmission of pathogens, thereby threatening other pollinating insects, especially wild bees (13, 14, 18–20). Indeed, many viruses originally found in honey bees and thus considered honey bee-specific viruses were shown to also infect several non-*Apis* bee species (see reference 21 and references therein). These results questioned the previously assumed strong host specificity for these viruses. So far, however, it has not been possible to determine whether these viruses have always circulated among pollinating insects or whether honey bees have spread the viruses; hence, knowledge of the directionality of interspecies virus transmission still remains elusive (22).

Another honey bee pathogen with presumed broad host specificity is the microsporidium *Nosema ceranae*. Microsporidia are fungus-related, obligate intracellular parasites infecting vertebrates and invertebrates alike. Three microsporidian species are well-known pathogens of bees. Western honey bees (*Apis mellifera*) are frequently found infected by the honey bee-specific microsporidium *Nosema apis* (23, 24), while *N. bombi* is a pathogen which is specific for bumblebees (*Bombus* spp.) (25, 26). *N. ceranae* was originally described as being specific for the Eastern honey bee (*A. cerana*) (27) but switched host from *A. cerana* to *A. mellifera* several decades ago (28–32). A recent report suggested that yet another host switch from *A. mellifera* to *Bombus terrestris* might have occurred (33). Since then, several field studies have detected *N. ceranae* not only in *B. terrestris* but also in other species of the genus *Bombus* (14, 34–38) and even in solitary bees such as *Osmia* spp. and *Andrena* spp. (39, 40). These studies indicated that *N. ceranae* is infective for bees in general (Apiformes) and may be a source of emerging infectious disease (EID) not only in *A. mellifera* but also in different wild bee species. In the case of *N. ceranae*, the directionality of interspecies transmission seems to be clear, with *A. cerana* and *A. mellifera* being the sources for infection in non-*Apis* bees (14).

As obligate intracellular parasites, microsporidia exist outside cells only as metabolically inactive spores which represent the infective stage of this pathogen. For *Nosema* spp., the infection cycle in bees has been elucidated in detail. After being ingested by an adult bee, the spores of *Nosema* spp. germinate in the host's midgut, thereby extruding the polar tube which initially perforates and thereafter supports transfection

of the epithelial cell (41–44). The injected sporoplasm develops and proliferates within the host cell into new mature spores through different developmental phases (merogony and sporogony), a process which takes about 96 h (41). Once the development is completed, cell lysis occurs and millions of fresh, infectious spores are released into the gut lumen and are excreted via defecation (45, 46), enabling the fecal-oral transmission route for this pathogen.

Severe *N. ceranae* infections can result in diarrhea, forcing the bees to defecate inside the hive (47). Such an outbreak of the disease nosemosis causes feces that contain infectious spores to spoil frames and combs (47). The infected feces promotes disease transmission within the colony, because bees engaged in ridding the hive of fecal remnants ingest these spores and become infected. If bees suffering from nosemosis fly out and defecate outside the hive, feces containing infectious *N. ceranae* spores might end up on flowers. This might be the case also when infected honey bees that do not yet suffer from dysentery show normal defecation behavior, i.e., defecate outside the hive. In both cases, infectious *N. ceranae* spores contaminating flowers might be picked up not only by another honey bee (intraspecies transmission, between-colony transmission) but presumably also by any flower-visiting insect, resulting in the described interspecies transmission of *N. ceranae* from *A. cerana* to *A. mellifera* and from there to other non-*Apis* bees. In this context, infection of bumblebees with *N. ceranae* is particularly worrying, because in some cases, dramatic declines in abundance and species richness of *Bombus* across continents have been reported previously (11, 48–51). Although a definite link between these declines and emerging pathogens such *N. ceranae* (14, 33–37) has not been established yet, it cannot be ruled out entirely. Therefore, controlled experimental studies are urgently needed in order to be able to better assess the danger that *N. ceranae* infections pose for bumblebees.

Here, we present our results from analysis of the impact of *N. ceranae* on individual bumblebees. We performed controlled infection experiments by inoculating caged bumblebees (*B. terrestris*) with different dosages of viable *N. ceranae* spores. Infection of caged honey bees (*A. mellifera*) served as a positive control, whereas mock infection of bumblebees and honey bees served as a negative control. We monitored survival rates and determined individual infection status by microscopic examination of squash preparations, by PCR-based detection of *N. ceranae* DNA, and by histology as well as species-specific fluorescence *in situ* hybridization (FISH) analysis of midgut sections. Furthermore, the passage of ingested spores through the digestive tract of bumblebees and honey bees was followed over 24 h. The obtained data do not support that *B. terrestris* is a true host for *N. ceranae* but rather reinforce that PCR-based detection of microsporidia is generally not sufficient to prove infection (52, 53).

RESULTS

First experiment: comparative experimental inoculation of caged adult *Bombus terrestris* and *Apis mellifera*. To evaluate the potential of the honey bee pathogenic microsporidium *N. ceranae* to also act as pathogen of bumblebees, we examined the infectivity of *N. ceranae* spores in *Bombus terrestris* in controlled inoculation experiments. Infection of honey bees (*Apis mellifera*) with the same spore preparation served as a control for the infectivity of the spore suspension used. We did not find significant differences between the survival curves (Fig. 1) of spore-inoculated and control bumblebees (log rank test, P value = 0.10) and the survival curves of the inoculated honey bees and the control bees (log rank test, P value = 0.54). Comparing the survival curves of the inoculated bumblebees and the inoculated honey bees also did not reveal any significant difference (log rank test, P value = 0.28). We next analyzed the survival rates at day 14 postinoculation (p.i.) and did not find a statistically significant difference (chi-square test, P value = 0.10) between bumblebees inoculated with 50,000 *N. ceranae* spores (95.9% ± 3.5% [mean ± standard deviation {SD}]) and the mock-inoculated control bumblebees (86.7% ± 12.6% [mean ± SD]) (Table 1). The survival rates of inoculated honey bees (88.3% ± 5.8% [mean ± SD]) and control honey bees (91.7% ± 2.9% [mean ± SD]) did also not differ significantly (chi-square test, P

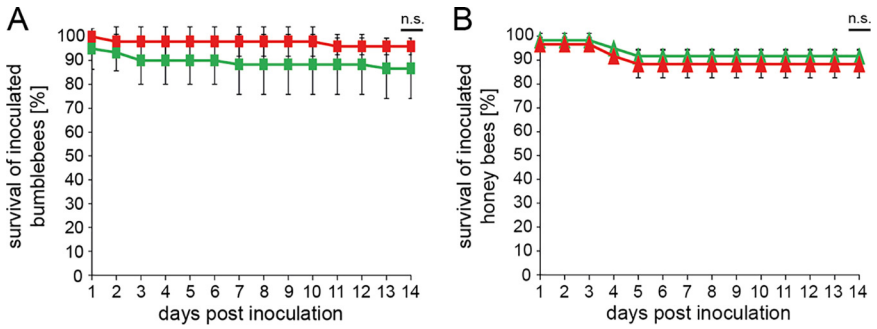

FIG 1 Survival of *B. terrestris* and *A. mellifera* inoculated with *N. ceranae* spores. The experiments were perfomed in triplicate for each group. Newly emerged bumblebees ($n = 16$, 16, and 17, red rectangles) (A) and honey bees ($n = 20$ each replicate, red triangles) (B) were individually inoculated with 5 μl sucrose/pollen solution (50% sucrose and 15% pollen) containing 50,000 viable *N. ceranae* spores. Control bumblebees ($n = 20$ each replicate, green rectangles) (A) and honey bees ($n = 20$ each replicate, green triangles) (B) were mock inoculated with 5 μl of sucrose/pollen solution (50% sucrose and 15% pollen) containing no spores. Survival was monitored daily over a time period of 14 days. Values are given as means ± SD. Statistical analysis of the survival curves was performed with the log rank test and revealed no statistically significant differences (P value = 0.10 for bumblebees [A] and P value = 0.54 for honey bees [B]). Survival rates at the end of the observation period (14 days p.i.) also did not differ significantly (n.s.) (chi-square test, P value = 0.10 for bumblebees and P value = 0.54 for honey bees).

value = 0.54), indicating that neither the survival of bumblebees nor that of honey bees was negatively affected by orally feeding them with 50,000 *N. ceranae* spores per bee.

We next examined the *N. ceranae* infection status of all bumblebees and honey bees that died in the course of the experiment or survived and were sacrificed at day 14 p.i. We did not detect any vegetative forms or spores of *Nosema* spp. in squash preparations of the midgut and Malpighian tubules of the spore-inoculated or mock-inoculated bumblebees. Accordingly, the infection rate as determined by microscopic analysis was 0% ± 0% (mean ± SD) (Table 1).

In contrast, we detected *Nosema* spores in the midgut squash preparations of all *N. ceranae*-inoculated honey bees; no vegetative forms or spores of *N. ceranae* were detected in any of the control honey bees (Table 1). Therefore, inoculation of naive newly emerged honey bees resulted in an infection rate of 100% ± 0% (mean ± SD) (Table 1), as expected. This result also confirmed that the spore preparation used for inoculation was infective, although no negative effect on honey bee survival was observed over the 14-day observation period (Fig. 1; see also Table 1).

TABLE 1 Experimental inoculation of newly emerged bumblebees and honey bees as well as 4-week-old and mixed-age bumblebees with freshly isolated, viable *N. ceranae* spores

Expt	Bee species	Total no. of tested individuals	*N. ceranae* spore dosage per individual	% infected (mean ± SD) by microscopy	% detected (mean ± SD) by PCR	Duration of expt (days)	% survival (mean ± SD)
1	*B. terrestris* (newly hatched)	49	50,000	0	6.13 ± 0.21	14	95.9 ± 3.5
		60	0	0	0	14	86.7 ± 12.6
	A. mellifera (newly hatched)	60	50,000	100 ± 0	100 ± 0	14	88.3 ± 5.8
		60	0	0	0	14	91.7 ± 2.9
2	*B. terrestris* (newly hatched)	60	6,500	0		28	68.3 ± 16.1
		60	50,000	0		28	66.7 ± 28.9
		60	500,000	0		28	75.0 ± 5.0
		73	0	0		28	83.6 ± 3.8
3	*B. terrestris* (four weeks old)	30	50,000	0		45	90.0 ± 0.0
		30	0	0		45	90.0 ± 10.0
	B. terrestris (mixed age)	60	50,000	0	0	45	43.3 ± 2.1
		23	0	0	0	45	8.3 ± 1.5
4	*B. terrestris* (newly hatched)	30	>5,550,000	0	0	43	70.0 ± 0.0

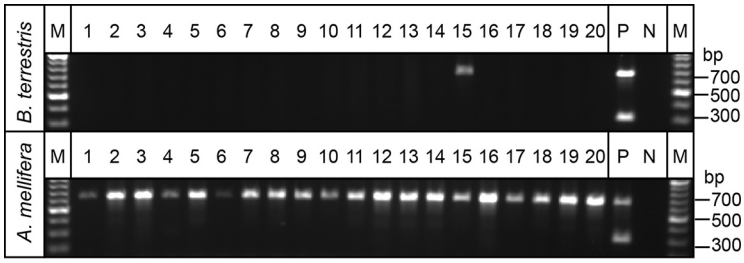

FIG 2 Molecular detection of *N. ceranae* in orally inoculated bumblebees and honey bees using PCR. Newly hatched *B. terrestris* and *A. mellifera* workers were orally inoculated with 50,000 *N. ceranae* spores each, and molecular detection of *N. ceranae* via PCR in inoculated animals was performed 14 days p.i. Representative results of 20 individuals are presented for both honey bees and bumblebees. (*P* = positive control, N = negative control, M = 100-bp gene ruler).

It was reported previously that up to 100% of bumblebees (*B. terrestris audax*) experimentally inoculated with *N. ceranae* spores tested positive for *N. ceranae* via PCR (37). Therefore, we additionally examined all surviving bumblebees and honey bees, as well as those animals that died during the experiments, using a duplex PCR protocol for detecting *N. ceranae* and *N. apis* (54). All mock-inoculated control bumblebees and honey bees tested negative for *Nosema* spp. by PCR (Table 1), indicating that the bumblebees and honey bees used did not carry a preexisting *N. apis* or *N. ceranae* contamination or infection. Of the inoculated bumblebees, only 6.13% $\pm$ 0.21% (mean $\pm$ SD) tested positive for *N. ceranae* by PCR (Table 1; see also Fig. 2). In contrast, we detected *N. ceranae* but not *N. apis* via PCR in all inoculated honey bees (100% $\pm$ 0%), substantiating the *Nosema* infection rate of 100% for the honey bees as determined by analysis of the squash preparations and confirming the identity of the pathogen to be *N. ceranae* (Table 1; see also Fig. 2).

To rule out the possibility that we missed a transient *N. ceranae* infection of bumblebees by determining the infection status after only 14 days, we analyzed Giemsa-stained midgut tissue sections of bumblebees and honey bees from independent inoculation experiments (50,000 *N. ceranae* spores per bee) at three time points, i.e., before inoculation and at 96 h as well as 240 h postinoculation. Histological analysis of the midgut tissue sections of bumblebees did not reveal any intracellular vegetative forms or newly generated spores of *N. ceranae* in the midgut epithelium at any time point (Fig. 3). However, we detected intracellular vegetative forms of *N. ceranae* in the midgut in all of the inoculated honey bees at 96 h p.i., and the epithelial cells were filled with environmental spores at 240 h p.i. (Fig. 3).

In Giemsa-stained tissue sections (Fig. 3), occasional *Nosema*-infected cells might be overlooked, particularly in the early stages of infection when spores are not yet present in the host cells. To overcome this limitation, we next applied a more sensitive analytic method, fluorescence *in situ* hybridization (FISH), and used *Nosema*-specific molecular probes (41) to analyze tissue sections of the alimentary tracts of the bees. A time series over 240 h (Fig. 4) confirmed the results obtained thus far and substantiated clear differences between bumblebees and honey bees. In the tissue sections, which were prepared from honey bees and bumblebees prior to inoculation (Fig. 4), no signal for *N. ceranae* was detected, again demonstrating that both the honey bees and bumblebees did not carry a preexisting *Nosema* infection. In the tissue sections of the bumblebee midguts, no signal for *Nosema* spp. was detected in the epithelial cells at any sampling time point (Fig. 4). However, distinct positive signals for *Nosema* spp. were detected in midgut epithelial cells of honey bees at 72 h p.i. and at every subsequent sampling time, with clear spore-shaped forms seen at 96 h p.i. and until 240 p.i. and with increasing numbers of infected cells and *Nosema* equivalents over time (Fig. 4). These results confirmed that no *N. ceranae* infection had developed in the midgut epithelium of bumblebees that had orally ingested 50,000 *N. ceranae* spores, which is a transmission route and dosage that resulted in a 100% infection rate in honey bees (Table 1; see also Fig. 2 to 4).

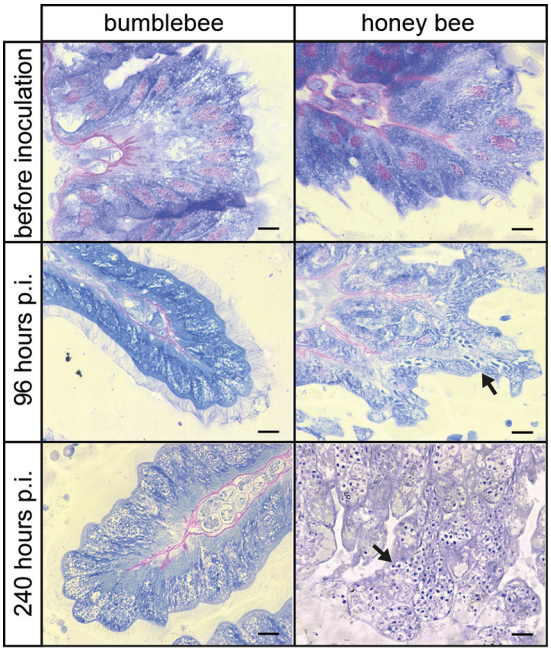

FIG 3 Histological analysis of midgut sections of bumblebees and honey bees inoculated with *N. ceranae* spores. Midgut tissue sections of *B. terrestris* and *A. mellifera* inoculated with 50,000 spores per bee were Giemsa stained and microscopically analyzed for the intracellular presence of *N. ceranae* vegetative forms (meronts) or environmental spores. Newly emerged bumblebees and honey bees were free of *Nosema* spp. prior to inoculation. No vegetative forms or environmental spores of *N. ceranae* could be detected in Giemsa-stained midgut sections of experimentally infected bumblebees at 96 or 240 h. In honey bees, *N. ceranae* meronts could be detected after 96 h (black arrow) and massive spore production in midgut epithelial cells was detected 240 h postinfection (black arrow). Representative pictures for the three time points are shown.

Second experiment: effect of *N. ceranae* spore dosage on the outcome of inoculation. The inability to experimentally infect bumblebees with *N. ceranae* was rather surprising considering the reports in the literature on *N. ceranae* infection of bumblebees (14, 33, 37, 38). However, the 100% infection rate obtained for the honey bees indicated that our spore preparation used for inoculation not only showed germination *in vitro* (see Materials and Methods) but was infective and, hence, that a lack of spore viability or infectivity in hosts could not be the reason for the unexpected result. However, spore dosage could be an issue because, in contrast to the dosage of 50,000 spores per honey bee or bumblebee used in our experiments thus far, previous studies reporting successful experimental infection of bumblebees had been performed by inoculating the bees with 6,500 spores per bee (37) or 100,000 spores per bee (14). Therefore, we next tested different dosages (6,500, 50,000, and 500,000 spores per bumblebee) in controlled inoculation experiments with newly emerged bumblebees in order to exclude the possibility that the initially used spore dosage of 50,000 spores per bumblebee was too low or too high and thus prevented a successful infection of the bumblebees. Mock-inoculated groups served as controls. In this experimental series, we also extended the observation period to 28 days to obviate the possibility that we had missed infection-related mortality due to the too-short duration of observation in the first experiment. At the end of the experiment, the survival rates of bumblebees that were inoculated with 6,500 spores each (68.3% $\pm$ 16.1% [mean $\pm$ SD]) or 50,000 spores each (66.7% $\pm$ 28.9% [mean $\pm$ SD]) or 500,000 spores each (75.0% $\pm$ 5.0% [mean $\pm$ SD]) or were subjected to mock inoculation (83.6% $\pm$ 3.8% [mean $\pm$ SD]) did not differ significantly from each other (chi-square test, P value = 0.10, Fig. 5), indicating that the survival rate of the bumblebees in the first experiment was not determined by the spore dosage used and that a dosage of even 500,000 *N. ceranae* spores per bumblebee had no negative effect on survival.

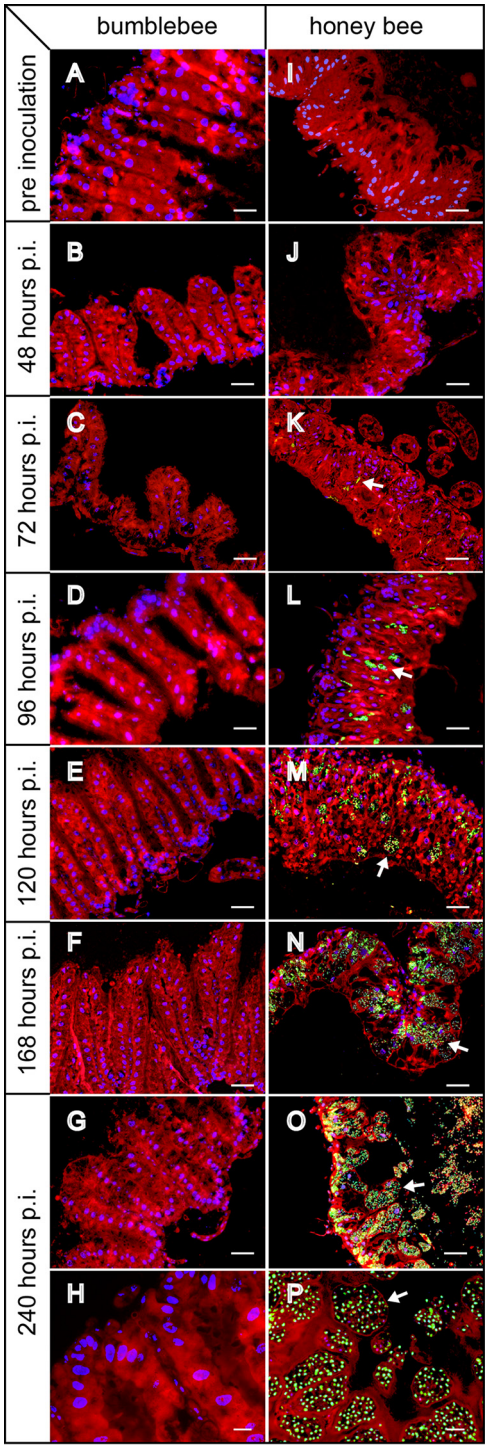

FIG 4 FISH analysis of midgut sections of experimentally inoculated bumblebees and honey bees. Representative results of *Nosema*-specific fluorescence *in situ* hybridization (FISH) analysis of tissue sections of *B. terrestris* (A to H) and *A. mellifera* (I to P) before inoculation and at 48, 72, 96, 120, 168, and 240 h p.i. Midgut sections were analyzed via FISH using a *Nosema*-specific 16S rRNA-targeted (green fluorescence) and a universal eukaryotic 18S rRNA-targeted (red fluorescence) oligonucleotide probe. Fluorescence signals were visualized by fluorescence microscopy at ×200 magnification (A to G and I to O) and at ×600 magnification (H and P). Eukaryotic nuclei were stained with DAPI (blue fluorescence). White arrows point to regions with signals that indicate *N. ceranae* infection of epithelial cells. Scale bars represent 100 μm (A to G and I to O) and 10 μm (H and P).

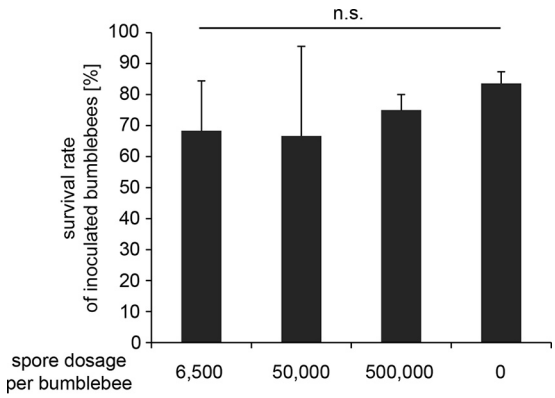

FIG 5 Survival rates of *B. terrestris* that had been inoculated with different spore dosages. Naive newly hatched bumblebees were orally inoculated with 6,500, 50,000, or 500,000 freshly isolated *N. ceranae* spores. For each spore-inoculated group, experiments were run in triplicate with 20 individuals. For the mock-inoculated group, we used 25, 25, and 23 individuals. Survival rate was analyzed daily for 28 days after inoculation, and data were analyzed by the chi-square test (*P* value = 0.10); n.s. = statistically not significant.

For analyzing the infection status of the inoculated bumblebees, squash preparations of the dissected midguts and Malpighian tubules of all control bumblebees and of all surviving and dead animals from the three dosage groups were microscopically analyzed. *N. ceranae* spores were detected neither in any of the control bumblebees (Table 1) nor in the midguts or the Malpighian tubules of any inoculated bumblebee (Table 1). These results indicated that the spore dosage was not the reason that the bumblebees did not become infected in the first experiment.

Third experiment: effects of bumblebee age and source on the outcome of inoculation. Another possible explanation for the failure to experimentally infect *B. terrestris* with *N. ceranae* could be the age of the bumblebees used in our experiments. While the use of newly emerged honey bees for experimental *Nosema* infection is well established and was 100% successful in our experiments (Table 1; see also Fig. 2 to 4), this age cohort might not be optimal for infection of bumblebees. Older bumblebees might be more suitable because interspecies transmission of *N. ceranae* from honey bees to bumblebees most likely occurs during foraging on shared flower resources and because foraging is a task which is seldom performed by newly emerged bumblebees.

Therefore, in the next series of experiments we used adult *B. terrestris* bees (4 weeks old) which we inoculated with 50,000 freshly isolated *N. ceranae* spores each; mock-inoculated bumblebees of the same age served as a control. This time, survival of the bumblebees was monitored over 45 days; thereafter, the surviving bumblebees were sacrificed. The survival rates of inoculated (90% $\pm$ 0% [mean $\pm$ SD]) and mock-inoculated (90% $\pm$ 10.0% [mean $\pm$ SD]) bumblebees (Table 1) were nearly identical (chi-square test, *P* value = 1.0). Analysis of the infection status of all spore-inoculated and mock-inoculated bumblebees was performed via microscopy of squash preparations of dissected midguts and Malpighian tubules. No microsporidian spores were detected in any midgut or Malpighian tubule preparation, resulting in a microscopically determined infection rate of 0% (Table 1).

We next investigated whether the source of the bumblebees might influence the outcome of our experiments. Thus far, we had obtained all bumblebees from one commercial bumblebee breeder (Koppert s.r.o., Nové Zámky, Slovakia), and therefore there is the possibility that a specific (though not defined) genetic background of the bumblebees might have influenced the experiments. So we decided to perform another experiment and to use *B. terrestris* colonies from a different commercial bumblebee breeder (Biobest Group NV, Westerlo, Belgium) which provided the bumblebees for a recently published study (14). From these colonies, we obtained 83 mixed-age bumblebees and individually inoculated 60 of them (three technical replicates with 20 individuals per

TABLE 2 Microscopic detection of *N. ceranae* spores in honey stomach, midgut, or hindgut of bumblebees and honey bees at different time points postinfection

	N. ceranae spore detection					
	Honey stomach		Midgut		Hindgut	
Time point	*Bombus terrestris*	*Apis mellifera*	*Bombus terrestris*	*Apis mellifera*	*Bombus terrestris*	*Apis mellifera*
10 min p.i.	+	+	+	+	−	−
30 min p.i.	+	+	+	+	−	−
2 h p.i.	+	−	+	+	−	−
6 h p.i.	−	−	+	+	+	−
12 h p.i.	−	−	−	+	+	−
24 h p.i.	−	−	−	+	+	+

replicate) with 50,000 spores each; 23 mock-inoculated bumblebees (three technical replicates with 8, 8, and 7 individuals per replicate) served as a control (Table 1). The survival rates of spore-inoculated (43.3% ± 2.1% [mean ± SD]) and mock-inoculated (8.3% ± 1.5% [mean ± SD]) mixed-age "Biobest" bumblebees (Table 1) were significantly lower than the survival rates of the bumblebees of the other experiments (chi-square tests, P values < 0.0001). However, microscopic analysis of squash preparations of dissected midguts and Malpighian tubules of both the dead and surviving inoculated bumblebees 2 weeks p.i. again did not result in the detection of spores, indicating that none of the bumblebees carried an *N. ceranae* infection (Table 1). All mock-infected bumblebees were also free of *N. ceranae* infections (Table 1). These results were also substantiated by PCR analysis, which did not result in the detection of *Nosema* specific amplicons in any of the samples (Table 1).

The counterintuitive finding that *N. ceranae*-inoculated bumblebees had a higher survival rate than the mock-inoculated bumblebees is in agreement with the results shown previously by Fürst and coworkers (14). In that study, the bumblebees used in experimental infection assays were obtained from the same source (Biobest) and the *N. ceranae*-inoculated group survived 4 days longer than the control group.

Fourth experiment: effect of repeated inoculation of caged *B. terrestris* with *N. ceranae*. In a last and rather desperate attempt to enforce successful *N. ceranae* infection of bumblebees, we established a worst-case scenario with repeated inoculation of 30 bumblebees with *N. ceranae* spores combined with placement of the bumblebees into cages contaminated with the *N. ceranae* spore-positive fecal matter from *N. ceranae*-infected honey bees. Microscopic analysis of squash preparations of dissected midguts and Malpighian tubules of both the dead and surviving inoculated bumblebees at 43 days p.i. revealed no evidence of infection.

Retention time of *N. ceranae* spores in the digestive tracts of bumblebees and honey bees. In order to unravel the reasons for the lack of *N. ceranae* infection in experimentally inoculated *B. terrestris* bees, we comparatively followed the fates of the ingested spores in inoculated bumblebees and honey bees from 30 min to 24 h p.i. by classical microscopic analysis of squash preparations of the honey stomach, the midgut, and the hindgut (Table 2; see also Fig. 6). We detected spores in the honey stomach of bumblebees until 2 h p.i., whereas spores were detectable in the honey stomach of honey bees only until 30 min after oral uptake, demonstrating that both the bumble-

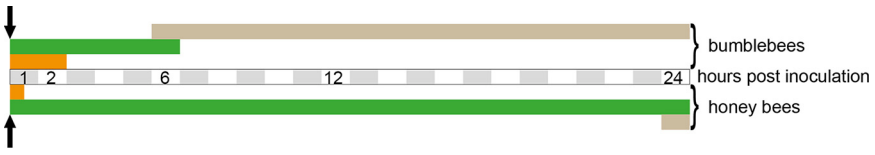

FIG 6 Passage of *N. ceranae* spores through the intestine of *B. terrestris* and *A. mellifera*. The presence of *N. ceranae* spores in the honey stomach (orange), midgut (green), and hindgut (brown) was determined by microscopic analysis of squash preparations prepared at 30 min, 2 h, 6 h, 12 h, and 24 h p.i. (time point of inoculation indicated by black arrow). For each time point, three individuals were analyzed.

bees and the honey bees had ingested the spores but that the spores left the honey stomach earlier in the honey bees than in the bumblebees. In the lumen of bumblebee midguts, *N. ceranae* spores were detected from 10 min until 6 h p.i. In contrast, *N. ceranae* spores were still detected in *A. mellifera* midguts after 24 h, suggesting a rather long retention time of the spores in the honey bees' midguts. Moreover, analyzing the hindguts revealed that in the bumblebees, spores were already detectable in the hindguts at 6 h after oral inoculation whereas in the hindguts of honey bees spores were not detected earlier than 24 h p.i. These results demonstrated that the retention time for orally ingested spores in the midgut, the primary tissue for *Nosema* infection, was only 6 h in bumblebees but was at least 24 h in honey bees; thus, the passage of the *N. ceranae* spores through the honey stomach and midgut was at least 18 h faster in bumblebees than in honey bees.

DISCUSSION

Emerging infectious diseases (EIDs), whether they are caused by viruses, bacteria, fungi, or parasites, are a major global threat to human, animal, and plant health and are contributing to species declines. Prerequisites for an emerging disease to become established are (i) that the infectious agent has to be introduced into a population of susceptible hosts and (ii) that the infectious agent has to have the ability to infect the new host, reproduce within the new host, and spread within the population. Therefore, host susceptibility and pathogen reproduction are the decisive features for the formation of a new host-pathogen interaction and should be proven before classifying an infectious agent as emerging for a new host.

Recently, the honey bee pathogenic microsporidium *N. ceranae* was reported to be an emerging infectious agent for *B. terrestris* as well as other bumblebee and wild bee species. Accordingly, we expected *B. terrestris* to show susceptibility to *N. ceranae* infection and we expected *N. ceranae* to reproduce within *B. terrestris*. To investigate this new host-pathogen relationship, we performed various infection experiments with caged *B. terrestris* bees of different ages and origins by the use of different *N. ceranae* spore dosages and incubation times. In the literature, at least two different spore dosages (6,500 and 100,000 spores per bumblebee) were described to be appropriate for successful infections of *B. terrestris* with *N. ceranae* (14, 37). We decided to start with 50,000 viable *N. ceranae* spores per bee because we aimed at high infection rates but also moderate mortality of infected bumblebees. Furthermore, dosages of 50,000 *N. ceranae* spores per bee were previously applied frequently and successfully when *A. mellifera* was used as host (55–57). We also used 6,500, 50,000, and 500,000 spores per bumblebee to span and exceed the range of spore dosages reported in the literature so far. Furthermore, we performed an experiment in which bumblebees were repeatedly inoculated with *N. ceranae* spores, resulting in an estimated dosage of 5,550,000 spores per bumblebee, and additionally exposed these bumblebees to infective fecal matter of *N. ceranae*-infected honey bees. We analyzed the infection status of the bumblebees using microscopy of squash preparations and PCR-based methods but did not find any evidence for *N. ceranae* infection of *B. terrestris*.

In susceptible hosts allowing the intracellular reproduction of *N. ceranae*, vegetative forms or newly produced spores of this parasite are found inside host cells and infectious spores are released from lysed host cells. For honey bees, countless studies have been reported which demonstrated infection of midgut epithelial cells. Therefore, we combined microscopy, histology, and FISH analysis in order to show that *N. ceranae* is able to infect *B. terrestris* cells. However, we have not found any spores in squash preparations of the midgut and Malpighian tubules and have not found any reproductive stages of *N. ceranae* inside host cells. Through a series of experiments, we ruled out the possibility that spore infectivity, spore dosage, incubation time, or age and source of the bumblebees caused these negative results, thereby clearly demonstrating that, in our experiments, no infection and production of new spores took place in *B. terrestris* after they ingested infectious *N. ceranae* spores. Therefore, our results question the

classification of *N. ceranae* as an emerging infectious agent for bumblebees as a new host.

In contrast to these results, an increasing number of studies have reported *N. ceranae* infections of bumblebees in the field (14, 20, 33–39). How can this be explained? It is conceivable that bumblebees are exposed to and even pick up *N. ceranae* spores when foraging on a flower that has been visited by an *N. ceranae*-infected honey bee. However, in order to qualify as a new host for *N. ceranae*, the uptake of spores has to initiate an infection in the new host, i.e., has to be followed by spore germination, successful infection of host cells, and production of new spores. If these events do not occur, ingested spores simply pass through the digestive tract and are defecated again without having established an infection. Therefore, for assessing whether *N. ceranae* is actually an emerging infectious agent that has expanded its host range and established a new pathogen-host relationship, the distinction between genuinely infected bumblebees and bumblebees that only carry spores that pass through the digestive tract is crucial.

Most studies on *N. ceranae* detection in bumblebees used PCR protocols for the detection of this parasite, and it is widely assumed that detection of *N. ceranae* DNA (fragments) indicates *N. ceranae* infection. However, PCR detection of *N. ceranae* DNA does not allow discrimination between infection and contamination (nongerminated spores passing through the digestive tract), as already discussed recently (52, 53). Hence, *N. ceranae* detection by PCR does not equal detection of *N. ceranae* infection. Consequently, the bumblebee studies in the past in which only PCR-based detection protocols have been used (14, 20, 33–36, 38, 39) should be treated with caution because they might have detected only ingested spores which are on their way through the digestive tract and which never germinated and successfully infected bumblebee host cells. Unfortunately, this overinterpretation of PCR-based *Nosema* detection is widespread in the literature and can even be found in high-ranked publications (14).

To the best of our knowledge, there has been only one study which combined molecular detection and microscopic detection of *N. ceranae* in field samples (37). In that study, it was reported that 21% of the sampled bumblebees (*Bombus* sp.) tested positive for *N. ceranae* via PCR but that *N. ceranae* was detected by microscopy in only 19% of that 21%; hence, among all of the bumblebees sampled, spores could be detected in only 4%. The spore counts were rather low, and no further methods to prove infection were applied; thus, knowledge remained elusive concerning whether these spores were the result of a true *N. ceranae* infection or were just passing through the digestive tract.

In the study already mentioned above (37), *B. terrestris* bees were also experimentally fed with *N. ceranae* spores and their infection status was analyzed via PCR and microscopy. Nearly 100% of the experimentally infected *B. terrestris* bumblebees tested positive for *N. ceranae* DNA by PCR but only 4 of 50 experimentally inoculated bumblebees tested positive for *N. ceranae* spores. Therefore, there was again a considerable discrepancy between the molecular and microscopic results. Since no further methods to prove infection were applied, it is questionable whether the bumblebees were truly infected.

In our experimental study, less than 10% of the inoculated *B. terrestris* bumblebees tested positive for *N. ceranae* by PCR and no spores could be detected in any of the bumblebees when the bees were analyzed at the time of death or at the end of the experiment. We therefore performed a comparative time course experiment over 24 h to follow the fate of the ingested spores in honey bees and bumblebees. The passage of the *N. ceranae* spores through the honey stomach and midgut was at least 18 h faster in bumblebees than in honey bees; the retention time in the midgut was only 6 h for bumblebees and at least 24 h in honey bees. These results suggested that the retention time of the spores in the midgut might play a role for the infection success of ingested *N. ceranae* spores. It is conceivable that the duration of the intestinal passage is influenced by the diets, which differed between the different studies: The sucrose

solution was either not supplemented with pollen (37) or supplemented with artificial pollen (Nektapoll) (14) or with natural pollen tested to be *Nosema* spp. free (this study). If the intestinal passage is slowed in bumblebees fed an inappropriate, pollen-less diet, this might enhance the chance of *N. ceranae* spores to germinate and infect a bumblebee midgut cell, explaining the few bumblebees found to carry spores in one study (37). Therefore, it would be interesting to investigate whether the nutritional status of bumblebees influences the infection success of *N. ceranae* and hence whether the supply with pollen in an increasingly poorer landscape influences the risk to bumblebees becoming infected with *N. ceranae*. In such a scenario, *N. ceranae* would still not be an EID *per se* of bumblebees but would rather be an infection that affects only starving and malnourished individuals. In such a case, it would not be the honey bees that would serve as Typhoid Mary for bumblebees (https://www.sciencemag.org/news/2014/02/deadly-virus-widespread-british-bumblebees) but rather the environmental conditions, which might indirectly enhance the susceptibility of some bumblebees to sporadic *N. ceranae* infections. Further studies are needed to address this issue and to identify the specific conditions which might enable *N. ceranae* to establish an infection in bumblebees.

In conclusion, the impression left by the many publications in the recent past concerning the allegedly frequent interspecies transmission of *N. ceranae* from honey bees to bumblebees has to be treated with caution. Most of the studies reported *N. ceranae* detection only by PCR; therefore, the authors were unable to rule out the possibility that they detected contamination rather than infection. The observed lack of infection might be influenced by the more rapid gastrointestinal passage in bumblebees in comparison to honey bees, although inappropriate conditions (e.g., suboptimal pH or lack of specific germination triggers) in the bumblebees' midgut might also negatively influence spore germination, thus preventing infection. But even if bumblebees are not true hosts for *N. ceranae* and do not become infected, they are still able to defecate ingested spores in new locations, contributing to the spatial spread of *N. ceranae*.

MATERIALS AND METHODS

Bee material. Newly hatched *B. terrestris* workers were obtained in batches of 100 bumblebees from the cooperating commercial bumblebee breeder Koppert s.r.o. (Nové Zámky, Slovakia). Upon arrival in the laboratory, all bumblebees were transferred from their transport packages into cages (20 individuals per cage) and incubated in climate chambers at 33.5°C with 55% relative humidity.

For one experiment (the 3rd experiment), *B. terrestris* workers were obtained from a commercial bumblebee breeder of Biobest Group NV (Westerlo, Belgium) and were supplied in standard hives containing mixed-age individuals. Upon arrival, the hives (with closed flight entrances) were placed in a climate chamber at 33.5°C with 55% relative humidity. For collection of bumblebee foragers, the flight entrances were opened and the bees were sampled from the flight entrances upon their trying to leave the hive. Collected bumblebees were caged (20 individuals per cage) and incubated in a climate chamber at 33.5°C with 55% relative humidity.

All honey bees used in this study were obtained from *Apis mellifera* colonies of the apiary of the institute (Institute for Bee Research, Hohen Neuendorf), which is located near Berlin, Germany. Brood frames with sealed brood were removed without nurse bees, and the frames were individually placed in a mesh walled cage and incubated overnight at 35°C. Newly hatched worker bees were caged (20 bees per cage) and were incubated in a climate chamber at 33.5°C with 55% relative humidity.

Honey bees as well as bumblebees were fed with a food solution containing 50% (wt/vol) sucrose syrup and 15% (wt/vol) pollen (provided *ad libitum*), which was replaced daily with freshly prepared solution until the end of the experiment. The sucrose syrup was supplemented with pollen in order to enhance the survival rate of the caged bees and also to increase spore intensities in infected animals (58). The pollen was obtained from a local distributor (Apispro, Hohen Neuendorf, Germany) and tested to be free of *Nosema* spp. via microscopy and PCR analysis (54, 59, 60).

All bee material was screened for viral and microsporidian infections prior to be used in the experiments. Twenty individuals from each batch (Koppert) or hive (BioBest) of bumblebees or each honey bee source colony were sampled and dissected to obtain the heads for virus analysis and the abdomens for detection of *Nosema* spp. The presence or absence of viral pathogens (deformed wing virus [DWV], sacbrood virus [SBV], acute bee paralysis virus [ABPV], and chronic bee paralysis virus [CBPV]) was determined as already described (61). In brief, RNA was extracted from heads of 20 individual bumblebees and honey bees using the RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RT-PCR was performed with a OneStep reverse transcription-PCR (RT-PCR) kit (Qiagen) according to standard protocols. Presence or absence of microsporidian infections was analyzed microscopically as already described (58). Briefly, 20 abdomens were macerated in 4 ml water

using a mortar and pistil and the homogenate was microscopically analyzed with a binocular microscope (Olympus, Hamburg, Germany) at ×400 magnification. For the experimental infections, only bumblebees and newly hatched worker honey bees that had originated from pathogen-free batches (bumblebees Koppert), hives (bumblebees Biobest), or colonies (honey bees of the institute's apiary) were used.

***N. ceranae* spore material.** For obtaining pure *N. ceranae* spore suspensions, *Apis mellifera* colonies from the institute's apiary located near Berlin, Germany, were used as source colonies. Colonies infected with *N. ceranae* but not with *N. apis* were identified via microscopy (spore detection in hindguts) followed by species-specific duplex PCR (species differentiation) essentially as already described (54, 59, 60). Only those colonies that were tested to be positive for *N. ceranae* infections but negative for *N. apis* or mixed infections were used for purification of *N. ceranae* spores as already described (41). Purified spore suspensions were tested again via species-specific duplex PCR (54) to rule out any contamination with *N. apis* spores. In addition, the germination capacity of each spore suspension was tested as already described (41, 60). Briefly, an aliquot of each spore suspension was air-dried on glass slides, germination was triggered by adding 30 μl of 0.1 M sucrose–phosphate-buffered saline (PBS) buffer, and the proportion of germinated spores was quantified microscopically. Only pure *N. ceranae* spore suspensions with high (>80%) germination rates were used for subsequent experimental infections.

Experimental infection of *A. mellifera* and *B. terrestris* with *N. ceranae*. For the experimental infections, all honey bees and bumblebees were starved for 4 h and subsequently fed individually with 5 μl sucrose/pollen food solution containing freshly isolated *N. ceranae* spores in different concentrations as outlined for each experimental series. Only bees that consumed the entire 5 μl spore-spiked food solution were included in the experiments, thus ensuring that the infection dosages were the same for all bees in the respective experiments. Survival of the bees was monitored daily over the duration of the respective inoculation experiments. Dead animals were removed, and their infection status was directly checked by microscopic analysis of squash preparations. At the end of the experiments, all survivors were sacrificed and their infection status was also microscopically analyzed. In addition, PCR-based protocols, histology of Giemsa-stained tissue sections, or fluorescence *in situ* hybridization (FISH) analysis was applied as described here for each experimental setup.

Four different series of inoculation experiments were performed as follows.

First experiment: comparative experimental inoculation of caged adult *B. terrestris* and *A. mellifera* bees. Naive *A. mellifera* ($n = 60$) and *B. terrestris* ($n = 60$) worker bees were individually inoculated with 50,000 spores each by feeding a 5-μl spore solution containing the respective spore dosage to each of them. As a control, bumblebees ($n = 60$) and honey bees ($n = 60$) were mock infected by feeding them the sucrose/pollen solution without spores. Unfortunately, we had to exclude 11 bumblebees during the experimental *N. ceranae* inoculation because they refused to consume the entire volume of the 5-μl spore solution. Therefore, the three replicates of the *N. ceranae*-inoculated bumblebees finally comprised 16, 16, and 17 individuals. In all other groups (*N. ceranae*-inoculated honey bees and mock-inoculated bumblebees and honey bees), each of the three replicates consisted of 20 individuals, resulting in $n = 3$ groups with 20 tested bees per group. Survival of orally inoculated bees was monitored daily over a time period of 14 days.

The infection status of all bumblebees and honey bees (dead during the observation period or sacrificed at the end of the experiment) was determined microscopically as well as by PCR-based methods. To this end, the dissected midguts of the bees were further dissected into two parts. One part of the tissue was used for preparing squash preparations, and the other part of the tissue was transferred into a PCR-clean reaction tube (Eppendorf) for DNA extraction and subsequent species-specific duplex PCR (54).

For specific *N. ceranae* detection in tissue sections via Giemsa staining and FISH analysis, a separate experiment was performed with 3 groups of 20 honey bees and 3 groups of 20 bumblebees inoculated with 50,000 spores per individual. Three bees (one bee per replicate) were removed from the experimental groups every 24 h over a time period of 10 days. For analysis, alimentary tracts from the honey bees and bumblebees were fixed in 4% Roti-Histofix (Roth, Karlsruhe, Germany) 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, 168 h, 192 h, 216 h, and 240 h p.i. Preparation of midgut sections, Giemsa staining, and *Nosema*-specific FISH analyses were performed as recently described (41, 61, 62) using an Eclipse Ti-E fluorescence microscope (Nikon, Düsseldorf, Germany) for analysis.

Second experiment: effect of *N. ceranae* spore dosage on the outcome of inoculation. In the second experiment, we individually inoculated newly hatched bumblebees ($n = 3$ groups of 60) with 6,500, 50,000, or 500,000 *N. ceranae* spores per bee. Three technical replicates with 20 bumblebees per replicate (3 groups of 20) were tested per dosage. The bees were caged (20 bees per cage) and incubated in a climate chamber at 33.5°C with 55% relative humidity. The mock-inoculated control group ($n = 73$) was fed with sucrose/pollen solution only and was divided into groups of 25, 25, and 23 animals per cage. The observation period ended at day 28 after oral inoculation. The infection status of all bumblebees was determined microscopically by analyzing squash preparations of midguts.

Third experiment: effects of *B. terrestris* age and source on the outcome of inoculation. In order to analyze the effect of bumblebee age on the outcome of inoculation, we incubated naive bumblebees in cages ($n = 20$ each cage) in a climate chamber at 33.5°C with 55% relative humidity for 28 days prior to oral inoculation. Thereafter, 30 adult bumblebees were inoculated individually with 50,000 spores each. As a control, 30 adult bumblebees were inoculated with sucrose/pollen solution only. Three replicates, with each group (inoculated and mock inoculated) consisting of 10 bees per replicate, were transferred to the climate chamber and incubated for another 45 days. The infection status of all bumblebees was determined microscopically by analyzing squash preparations of midguts.

In order to analyze the combined effects of bumblebee source and age on the outcome of inoculation, bumblebee colonies (Biobest Group NV, Westerlo, Belgium) harboring mixed-age bumblebees were used to sample 60 bumblebees directly from the flight entrances. Caught bumblebees were individually inoculated with 50,000 spores each and caged in cohorts of 20 bees per cage. Due to a shortage of available bumblebees appearing at the flight entrance in an adequate time frame, we were able to catch only 23 bumblebees for mock infection. These bumblebees were fed sucrose/pollen solution, and they were divided into three replicate groups consisting of eight, eight, and seven bumblebees per cage. All bumblebees were incubated in a climate chamber at 33.5°C with 55% relative humidity for 14 days. The infection status of the bumblebees (dead during the incubation period or sacrificed at the end of the experiment) was determined microscopically by analyzing squash preparations of midguts and by PCR detection of *Nosema* DNA.

Fourth experiment: effect of repeated inoculation of caged *B. terrestris* with *N. ceranae*. For the fourth experiment, we simulated a worst-case scenario for the bumblebees. We inoculated 3 groups of 10 newly hatched bumblebees with 50,000 spores each and incubated them for 7 days in the climate chamber at 33.5°C and 55% humidity with food supplied *ad libitum*. Subsequently, we again inoculated all 30 bumblebees with 500,000 spores each and incubated them for another 21 days with food supplied *ad libitum*. After this, we transferred the three groups of bumblebees into the three cages which had been used in the honey bee infection experiments and which were therefore spoiled with the fecal matter of *N. ceranae*-infected honey bees. In addition, the provided food solution (2 ml) contained 50,000,000 *N. ceranae* spores per ml and was replaced by nonspiked food solution only after the bumblebees had fully ingested the spiked food solution. The bumblebees were incubated for another 15 days. The infection status of the bumblebees (dead during the incubation period or sacrificed at the end of the experiment) was determined via microscopic analysis of squash preparations.

Squash preparations. Squash preparations have been described previously to be especially useful in the detection of microsporidian infections (63, 64) because this technique allows the detection of intracellular vegetative stages, spore-releasing cells, and released spores (59). Therefore, in order to evaluate the infection status of inoculated honey bees and bumblebees, squash preparations of all animals were microscopically analyzed for the presence of established infections in the midgut epithelial cells and Malpighian tubules. To this end, alimentary tracts of infected animals were removed from the abdomens by using forceps and the tissue was dissected into hindgut and midgut with adherent branching Malpighian tubules on 76-by-26-mm glass slides (Roth, Karlsruhe, Germany) by using a sterile scalpel (Roth, Karlsruhe, Germany) for each segment.

The midguts were further dissected dorsoventrally, and squash preparations (59) were generated from the anterior part of the midguts that included the Malpighian tubules by squeezing the tissues between a glass slide and a 24-by-60-mm cover slip (VWR, Darmstadt, Germany). The prepared slides were microscopically screened (at ×400 magnification) for the general presence of *Nosema* spores and especially for the presence of intracellular *Nosema* spores, proving the development of a true infection in host cells. Squash preparations which did not show any *Nosema* vegetative stages or spores resulted in the classification "not infected" for the respective individuals.

Species-specific duplex PCR. The posterior part of the midguts used for squash preparations was further processed for PCR analysis. To this end, each sample was transferred into a 1.5-ml reaction tube and mechanically crushed in a mixer mill (Qiagen) at 30 Hz for 30 s using a 3-mm-diameter tungsten carbide bead (Qiagen). DNA was extracted from the homogenized samples with the DNeasy plant minikit (Qiagen) according to the manufacturer´s protocol. A duplex PCR for specific detection of *N. ceranae* and differentiation between *N. apis* and *N. ceranae* was performed as recently described (54). Briefly, for species-specific detection of *N. apis* or *N. ceranae* on the basis of sequence differences in the DNA-dependent RNA polymerase II largest-subunit gene, primer pairs NosaRNAPol-F2/NosaRNAPol-R2 (5′-AGCAAGAGACGTTTCTGGTACCTCA-3′/5′-CCTTCACGACCACCCATGGCA-3′) and NoscRNAPol-F2/NoscRNAPol-R2 (5′-TGGGTTCCCTAAACCTGGTGGTTT-3′/5′-TCACATGACCTGGTGCTCCTTCT-3′) were used. PCR was performed by using a HotStarTaq Plus DNA polymerase kit (Qiagen) and 10 mM deoxynucleoside triphosphate (dNTP) mix (Peqlab, Erlangen, Germany) according to the protocols of the manufacturers. PCRs were performed with an initial DNA denaturation step at 95°C for 5 min followed by 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min and a final elongation step at 72°C for 10 min. Amplification products were separated in a 1% agarose gel, stained with ethidium bromide, and visualized under UV light.

Giemsa-stained tissue sections. For detection of intracellular vegetative forms and spores of *N. ceranae* at defined time points during the infection cycle, tissue sections from bumblebees and honey bees were prepared. The alimentary tracts were carefully removed with forceps, and the midguts were fixed in 4% paraformaldehyde for 24 h. Subsequently, the midguts were embedded in Technovit 8100 resin (Kulzer, Wehrheim, Germany) according to the manufacturer´s instructions. Histological semithin sections (3 μm) were prepared by using a rotary microtome (Thermo Scientific, Walldorf, Germany) and a tungsten carbide knife with a D profile (Leica, Wetzlar, Germany). All native histological sections were fixed on glass slides with tap water and stored at room temperature until further processing.

For visualization of infected tissues, Giemsa stain (Fluka, Thermo Scientific) (1:10 diluted in double distilled water) was used essentially as described in the manufacturer's instructions. After the incubation time of 10 min, the histological sections were washed with tap water and air-dried; for microscopy, a cover slip was mounted with Entellan (VWR).

Fluorescence *in situ* hybridization (FISH). FISH analysis was performed for specific detection of *Nosema* spp. in histological sections of bees according to the recently published protocol (41). Briefly, the oligonucleotide probe Nos16Srv (Texas Red; CTCCCAACTATACAGTACACCTCATA), labeled with Texas Red

fluorescent dye (Eurofins Genomics, Ebersberg, Germany), was used to produce red fluorescing signals of intracellular stages of *Nosema* spp. For contrasting bumblebee and honey bee host cells, a universal eukaryotic 18S rRNA-based oligonucleotide probe, Euk516 (fluorescein isothiocyanate [FITC]-ACCAGAC TTGCCCTCC [65]), labeled with FITC dye, was applied. For hybridization of each prepared histological section, 7.5 ng Texas Red-labeled oligonucleotide Nos16Srv and 10 ng FITC-labeled oligonucleotide Euk516 were diluted in 50 μl of hybridization buffer containing 20% formamide, 0.9 M NaCl, 20 mM Tris-HCl (pH 7.9), and 0.01% SDS. Hybridization was performed overnight at 46°C in humid chambers (Corning, Fisher Scientific, Schwerte, Germany). The hybridized tissue sections were then treated with DAPI (4′,6-diamidino-2-phenylindole)-methanol (1 mg ml^{-1}) for 10 min (blue fluorescence). Finally, tissue sections were washed, air-dried, and mounted with ProLong Gold antifade reagent (Fisher Scientific). Fluorescent microscopy was performed using an Eclipse-Ti fluorescence microscope (Nikon, Düsseldorf, Germany) with a standard set of fluorescence filters. For enhanced visualization of positive hybridization, the fluorescent signals were dye swapped by changing the settings in the NIS-elements software provided by Nikon. This means that a signal for the FITC dye (green) was swapped to red and that a signal for the Texas Red dye (red) was swapped to green.

Analysis of the retention time of *N. ceranae* spores in honey bees and bumblebees. For analysis of the retention time of spores in the honey stomach, midgut, and hindgut, another separate experiment was performed with 60 bumblebees and honey bees infected with 1,000,000 spores each as described above. In order to analyze the retention time of *N. ceranae* spores in the organs of the digestive tract after oral consumption, experimentally infected newly hatched bumblebees and honey bees were sacrificed at 10 min, 30 min, 2 h, 6 h, 12 h, and 24 h p.i. The honey stomachs, the midguts, and the hindguts were carefully removed from the animals at the respective time points, and the presence of spores in the respective organs was microscopically determined in three biological replicates by analyzing 10 visual fields at ×400 magnification.

Data analysis. Survival rates of experimentally inoculated honey bees and bumblebees at the end of the 1st, 2nd, and 3rd experiments were analyzed with the nonparametric chi-square test of independence. To compare the survival curves of inoculated honey bees and bumblebees over the time period of 14 days in the 1st experiment, the log rank test was used. For both tests, P values of <0.05 were defined as statistically significant. For all statistical analyses, XLSTAT Biomed statistics software (version 2018.4) was used.

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