# Biological effects of *Nosema ceranae* infections on honey bees (*Apis mellifera*) and bumblebees (*Bombus terrestris*)

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## Erklärung

Hiermit versichere ich, die vorliegende Dissertation mit dem Titel „Biological effects of *Nosema ceranae* infections on honey bees (*Apis mellifera*) and bumblebees (*Bombus terrestris*)“ selbstständig und ohne Benutzung anderer als der zugelassenen Hilfsmittel angefertigt zu haben.
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Die vorliegende Arbeit wurde in keinem früheren Promotionsverfahren angenommen oder als ungenügend beurteilt.

Potsdam, den 24. Mai 2022

Vivian Schüler

Die Dissertation wurde in englischer Sprache verfasst.

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# Contents

## Zusammenfassung

Honigbienen und Wildbienen sind bedeutende Bestäuber und tragen zu höheren Erträgen in der Landwirtschaft und zur Biodiversität der natürlichen Fauna bei. Jedoch führen Globalisierung und Industrialisierung von Bienenvölkern zu steigendem Infektionsdruck.
Globaler Handel und Transport von Bienen zur Maximierung des Ertrags bestimmter Trachten, beispielsweise Mandeln in den USA, führen zu erhöhtem Stress in den Völkern. Immer neue Umgebungen und Umwelteinflüsse, keine Zeit zum Adaptieren, einseitige Ernährung und die hohe Dichte von Bienenstöcken unterschiedlichster Herkunft bilden beste Bedingungen für einen hohen Austausch von Krankheiten und Parasiten zwischen den Bienenstöcken, aber auch mit den lokalen Bestäubern.
Das wohl schwerwiegendste Beispiel ist die ursprünglich aus Asien stammende Milbe *Varroa destructor*, die heute weltweite Verbreitung gefunden hat. *V. destructor* und zusätzlich die Pathogene die sie überträgt, wie beispielsweise das Flügeldeformationsvirus (DWV), setzen der Gesundheit der Bienenvölker zu.
Auch das ursprünglich aus Asien stammende, die östliche Honigbiene *Apis cerana* parasitierende Mikrosporidium *Nosema ceranae*, ist ein mittlerweile weltweit verbreiteter Parasit der westlichen Honigbiene *Apis mellifera*. Obwohl *N. ceranae*-Infektionen zu den häufigsten in Honigbienen zählen sind die Veröffentlichungen zu seiner Epidemiologie widersprüchlich. Auf der einen Seite wird *N. ceranae* gegenüber *N. apis* als virulenter, und als aufstrebender und tödlicher Krankheitserreger beschrieben, auf der anderen Seite kann ein Zusammenhang zwischen Völkerverlusten und *N. ceranae*-Infektion nicht bestätigt werden.
In meiner Dissertation habe ich einige dieser Widersprüche aufgegriffen um diese aufzulösen. Ich habe die Prävalenz von *N. ceranae* in Nordostdeutschland untersucht und konnte eine Saisonalität im Infektionsgeschehen beschreiben, welche bisher nur *N. apis* zugeschrieben wurde. Außerdem habe ich den Einfluss von *N. ceranae*-Infektionen im Herbst auf den Koloniestatus des Frühjahrs des Folgejahres untersucht und deren biologische Effektstärke berechnet.
Abschließend wollte ich die bisher unbekannten Parameter einer *N. ceranae*-Infektion bei Hummeln untersuchen (*Bombus terrestris*), welche zu dem Ergebnis geführt haben, dass Hummeln entgegen zahlreicher Veröffentlichungen keine wirklichen Wirte von *N. ceranae* sind.

## Abstract

Honey bees and wild bees are important pollinators and contribute to higher yields in agriculture and to the biodiversity of the natural fauna. However, globalization and industrialization of bee colonies lead to increasing infection pressure.
Global trade and transport of colonies for maximizing the yield of a specific crop (e.g. almonds in the USA) leads to stress: Quick change of environments and environmental influences, no time to adapt, one-sided nutrition and high population densities of bee colonies from different parts of the world resulting in an interchange of diseases and parasites between the beehives, but also with the local pollinators.
The most severe example is the mite *Varroa destructor*, which originates from Asia and is now found worldwide. Likewise, the pathogens that it vectors, e.g. deformed wing virus (DWV) affect the health of the bee colonies. Also from Asia is the originally *Apis cerana* parasitising honeybee pathogenic microsporidium *N. ceranae*, which parasitises Western honey bees *Apis mellifera* worldwide. Although it is one of the most common infections, publications on its epidemiology still seem to be contradictory. On the one hand *N. ceranae* is considered to be more virulent than *N. apis* and an emergent and deadly pathogen, on the other hand it has been described that there is no significant correlation between *N. ceranae* infection and colony losses.
Within my dissertation I addressed several of these contradictions and tried to shed light on ambiguities. I investigated the prevalence of *N. ceranae* in Northeast Germany and was able to describe a seasonality, which has been ascribed only to *N. apis* before. Furthermore, I investigated the influence of *N. ceranae* infections occurring in autumn on the colony status of the spring of the following year and calculated its biological effect size.
Finally, I wanted to investigate the so far unknown parameters of an *N. ceranae* infection in bumblebees (*Bombus terrestris*), which led to the conclusion that, contrary to numerous publications, bumblebees are not true hosts of *N. ceranae*.

## List of Abbreviations

| | |
|---|---|
| ***A. cerana*** | *Apis cerana* |
| ***A. mellifera*** | *Apis mellifera* |
| **ATP** | Adenosine triphosphate |
| **DCA** | drone congregation area |
| **DWV** | Deformed wing virus |
| **e.g.** | *exempli gratia* |
| **FISH** | Fluorescence in situ hybridization |
| ***G. mellonella*** | *Galleria mellonella* |
| **i.a.** | *inter alia* |
| **i.e.** | *id est* |
| **mRNA** | messenger ribonucleic acid |
| ***N. apis*** | *Nosema apis* |
| ***N. ceranae*** | *Nosema ceranae* |
| **PCR** | polymerase chain reaction |
| **p.i.** | post infection |
| **RFLP** | restriction fragment length polymorphism |
| **rRNA** | ribosomal RNA |
| **RT-qPCR** | quantitative reverse transcriptase PCR |
| **spp.** | *species pluralis* |
| ***V. destructor*** | *Varroa destructor* |

# 1 Introduction

What would humankind be without the honey bee? It is quite interesting how close our own history is entangled with these impressive insects.
In early history mankind started to domesticate bees because honey bee products, such as honey and bee wax, were desired. Since the early ages the medical properties of honey are well-known and since the Middle Ages in Europe honey bees wax was highly valued e.g. in abbeys for production of candles allowing independence from sunlight.
Nowadays it is possible to produce wax synthetically and electrical lighting replaced candles in most places. In modern medicine the antibiotics drastically reduced and even replaced the demand for honey in the medical sector and industrial refined sugar is used as sweetener. Put in a nutshell, humankind believes the support for human development lies in industrialization.
Even agriculture and apiculture were industrialized which had the goal to create higher yield but created unhealthy living conditions for pollinators leading to the worldwide problem of pollinator declines. However, the pollination service especially of insects is essential for an increased yield of many crop plants. This higher yield is mandatory to ensure an adequate global food supply and to allow humans a varied diet.
The industrialized agriculture and apiculture cause conditions that allow an easy spreading of pest and pathogens because too many hives are too close together. Also the hives get weakened due to pesticides used in agriculture and due to monocultures that lead to malnutrition (Shanahan, 2022).

## 1.1 Commercial Pollination

The sexual reproduction process of plants is called pollination, where pollen of the male anthers is transported to the female stigmata. One distinguishes between self-pollination, which is differentiated into autogamy and geitonogamy, and cross-pollination (xenogamy). In autogamy pollen are transferred within the same flower whereas in geitonogamy they are transferred to another flower of the same plant. Hence, xenogamy refers to the transmission of pollen to flowers of different plants of

the same species (Rhodes, 2018).
In contrast to pollination by wind and water, about 90% of flowering plants depend on pollination by animals, that vector the pollen (Ollerton et al., 2011). Besides honey bees 200.000 different species had been reported to be involved in plant pollination, including birds, bats and other insects like butterflies, wasps, moths and beetles (Rhodes, 2018).
It has been shown that a great variety of different pollinators is important for a healthy ecosystem (Garibaldi et al., 2013). Especially in the focus of climate change, biodiversity in both, managed and wild pollinators, is widely believed to be the best way to face the coming changes (Rader et al., 2013).
Hence, for mankind stable and intact ecosystems are essential to secure the global food and seed supply, because about 70% of the world's most important food crops show a higher yield in fruit and seed production when pollinated by animals (Klein et al., 2007). Also the yield increase depends on the number of different pollinators (Brittain et al., 2013). Especially vegetables, fruit and nuts, which humans demand for a balanced and healthy nutrition, are cross-pollinators and their yields directly dependent on animal pollination (Khalifa et al., 2021).
A study regarding almond pollination (Lundin et al., 2017) supports the hypothesis that the yield amount of crops benefits from an unharmed ecosystem and a high plant diversity. The results show that wild plants, that had been planted close to the almond orchard, did not diminish the pollination performance of honey bees by being distracted by the flowers of the wild plants. On the contrary, flower visits by honey bees doubled and wild pollinators that got attracted by the flowers of the wild plants also pollinated the crops.
Because the intensive and industrialized agriculture is far from being pollinator-friendly, sometimes the only solution to secure pollination is commercially used human-bred bees.
In account of their pollination performance honey bees are the preferred managed bees. In Germany they are considered the third most important economic livestock for humans, alongside with cattle and pigs (Böcking, 2010). And indeed, studies suggest they are the economically most important pollinators (Klee et al., 2007; Potts et al., 2016). Since the awareness of the importance of the honeybee as pollinator for crops, the number of managed hives has increased in the last half-century by ∼45% because they are cheap and easy to breed and keep. But since mankind and its demands for food keep growing the number of needed pollinators is constantly increasing (Aizen &

Harder, 2009).
As an alternative to the honey bee, other pollinating insects are bred by humans. There are bumblebees (e.g. *B. terrestris*) which are very efficient pollinators that can be reared and used as managed pollinators. Honey bees and bumblebees differ in their way of pollination and can be used together to achieve a higher pollination efficiency of e.g. blueberries as shown recently by (Miñarro & García, 2021) in Spain. Other established managed pollinators that are used commercially are stingless bees of the genus *Melipona* (Sommeijer, 1999) (Cortopassi-Laurino et al., 2006), leaf-cutting bees (*Megachile rotundata*) (Bosch & Kemp, 2002) or mason bees (*Osmia* spp.) (Sedivy & Dorn, 2014).
To make the appreciation for pollinators that drive this work more accessible to non-specialists the next sections present them in more detail. Honey bees and bumblebees are going to be introduced and characterized and their differences will be pointed out.

### 1.1.1 Western honey bee (*Apis mellifera*)

Honey bees are social insects of the order Hymenoptera which belong to the family Apidea. They live monogenic together in colonies, i.e. the female workers stay with one queen. All individuals of the two female castes form a superorganism where they perform harmonized tasks, determined by age polyethism comparable to cells in an organism. Because of the complex social structure of honey bees, they are called eusocial. This kind of structure is beneficial e.g. for division of labour, group protection against predators and "social immunity" (Cremer et al., 2007; Southwick & Moritz, 1992).
Initially, the genus *Apis* existed only in Europe, Asia and Africa, and was not distributed on the American continents and Australia. Due to human influence and globalization *Apis* can now be found worldwide. There are several other species than the Western honey bee *A. mellifera*. In Asia there are at least six further species of which in our context *Apis cerana* should be emphasized.

#### Anatomy

As with all insects, the body can be divided into three segments: head (caput), chest (thorax) and abdomen. The head is characterized by two compound eyes and three point eyes on the forehead as well as mouthparts (the mandibles) for chewing and licking and two antennae for tasting and feeling. The chest has three pairs of legs,

two pairs of wings and the musculature for movement. The vital internal organs, i.a. the digestive system and the fat body, as well as the stinger of the females are in the abdomen.
The body is protected by an exoskeleton made of chitin and sclerotin, and most of their body is covered with hair. Most hairs function as sensory organs (mechanoreceptors) and allow the bees to smell, taste and feel (temperature, vibration) (Sutton et al., 2016; Thurm, 1964). Bees do not have ears, but can perceive sound waves using mechanoreceptors on their hind legs and antennae. Furthermore, the hair helps the pollen stick to the bee i.a. because of the electric charge: bees are positively charged and blossoms are negatively charged (Clarke et al., 2017; Zakon, 2016). Honey bees have a corbicula ("pollen basket"), formed by a structural alteration of the hind legs (Rhodes, 2018).
Their nervous system is on the abdominal side. Honey bees have an open circulatory system without blood vessels where the haemolymph circulates and is being pumped to the head and flowing back down the ventral side to the abdomen.
The gut and the reproductive organs are encircled by the fat body, which is the major organ for energy storage and utilization and intermediary metabolism. It is a relative large organ, arranged as a loose tissue with thin lobes, so that it is maximal surrounded by haemolymph. This structure guarantees the quick availability of large amounts of energy, enabling them to fly (Arrese et al., 2001; Arrese & Soulages, 2010; Dettner & Peters, 2010). In addition, Strachecka et al. 2021 found the fat body in compartmented segments with individual functions and conclude that the fat body can be compared to the liver, pancreas, spleen and adipose tissues of vertebrates.

**Digestive System**

The digestive tract of adult bees is divided into three compartments, which are called foregut, midgut and hindgut (Chapman, 1998) (Figure 1).

Food is ingested via the mandibles or the sucker and passes over the oesophagus into the crop, also known as honey stomach. The crop is a muscle that can strongly dilate to be able to transport as much liquids, like nectar or water, as possible while the proventriculus prevents the nectar to be passed to the further digestive organs. In the hive, the content of the crop is regurgitated and passed on to other bees. For its own supply, the bee is able to pass on some nectar through the proventriculus into the midgut. As digestion takes place here, this area is also called the ventriculus. The food is digested by the help of secreted enzymes and resorbed by the cells of the

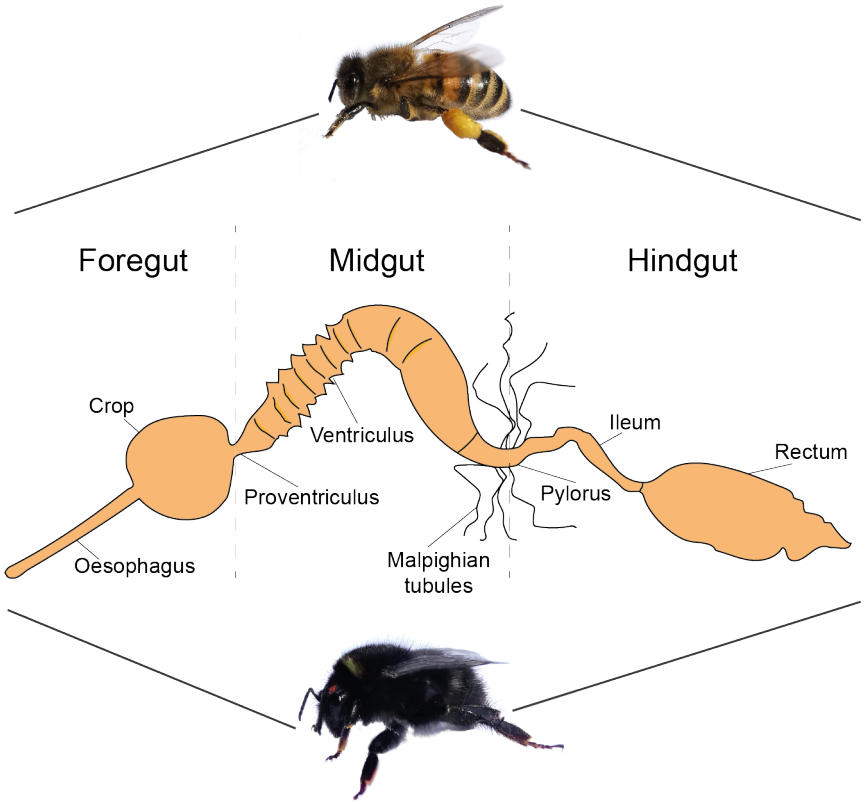



Figure 1: Scheme of the compartments of the digestive system of adult honey bees and bumblebees. Food is ingested and passed through the oesophagus into the crop. Food passing the proventriculus is digested in the midgut and move further to the hindgut where remains are accumulated in the rectum for defecation. Figure was adjusted to Kwong and Moran 2016.

epithelial layer. The peritrophic matrix protects the epithelial cells from mechanical and chemical damage and from pathogens. It is a multilayered lamellar structure composed of chitin, microfibrils, proteins, glycoproteins and proteoglycans, which are synthesized by the epithelial cells.

Digestive residues move further into the hindgut via the pylorus, a connection between large and small intestine. In front of the pyloric valve the Malpighian tubules are coupled, which function as bee's kidneys and filter the haemolymph via a system of about 100 single tubules. Indigestible residues are collected in the rectum. Honey bees defecate outside the hive, so during winter they retain the feces until warmer temperatures allow them to leave the hive (Aupperle & Genersch, 2016; Dettner & Peters, 2010; Engel & Moran, 2013).

**Developmental stages and different casts of the honey bee**

Larvae hatch from eggs in brood cells and pass various larval stages to become pupae. The pupae develop into nymph stage and finally to adult bees. The complete metamorphosis (holometabolism) takes 16 days for queens, 21 days for workers and 24 days for male bees, the drones. Embryonic development occurs during the three-day egg period. Depending on which of the three castes develops, the subsequent coiled larva stage lasts five days for queens, six days for workers or seven days for drones. The larva grows and moults four times, as the surrounding chitinous cuticle cannot grow. It develops into a stretched larva and when the brood cell is capped the larva spins itself into a cocoon with the sap of the spinning gland. Meanwhile, the metamorphosis to the adult bee takes place over several pupal stages and two more moults. The bee hatches by biting through the cell lid (Winston, 1987).

Honey bee queens develop from a fertilized egg. The hatched larva is fed exclusively with royal jelly and five days after the adult queen hatched, she is ready to leave the hive for the nuptial flight. Queens do the so-called nuptial flight only once in a lifetime and often fly relatively far to drone congregation areas (DCAs) and mate with up to 20 drones (Bastin et al., 2017). The sperm is collected in the queens' spermatheca (*receptaculum seminis*) which serves her whole life. The queen can actively control the fertilization of the eggs depending on the size of the brood cells and lays either fertilized eggs from which diploid worker bees or young queens hatch or non-fertilized eggs from which haploid drones hatch.

The queen's abdomen is larger than that of the workers, because only she has fully developed ovaries in which eggs can mature. Queens can lay up to 2,000 eggs per day (Nolan, 1925). If the queen can no longer provide the necessary offspring the workers replace her. Young queens are formed in queen cells at the edge of the comb surfaces. The first hatched young queen kills her rivals. The old queen either moves out with part of the worker bees forming a swarm or is stabbed by the workers. In temperate climates, honey bee workers can be classified as short-lived summer bees or long-lived winter bees. The activities of summertime worker bees are clearly defined. They start their work in the hive as cleaning bees ($1^{st}$ to $3^{rd}$ day). Later they take care of the brood as nurse bees (day 4 to 10). For further 10 days they are construction bees building honeycombs, but also guard the entrance. When at least 21 days old they leave the hive as foragers to collect nectar and pollen and fetch water(Aupperle & Genersch, 2016). In order to ensure colony survival in winter, workers build up stocks by storing resources, including huge honey stores. A bee colony can consist of 30,000

to 80,000 workers in summer. Their life-span is around 4-6 weeks. In contrast, the amount of workers decreases to a colony size of 10,000 to 20,000 individuals in winter (van Nerum & Buelens, 1997). After the foraging period, the shortening of pollen leads to the physiological transition into long-lived winter bees with a lifespan of around 6 to 8 month (Amdam & Omholt, 2002). Their bodies store fats and proteins destined to feed the first generation of worker brood in spring. To hibernate, winter bees form an active cluster that ensures thermoregulation and queen-care (Knoll et al., 2020; van Nerum & Buelens, 1997).
As drones are, from an energetic point of view, needless in winter, they do not hibernate and are displaced and killed by the worker bees in fall. In temperate climates, drones are produced during spring and summer month, when honey bee colonies can support them and when the virgin queens are available for mating (Winston, 1987). Their physical appearance differs from the workers primarily in their larger compound eyes and a short, broad abdomen. Interestingly, their legs are not designed to transport pollen and their sucker is atrophied so they have to be fed. They leave the hive when reaching sexual maturity (12 days after hatching) and encounter young queens for mating at DCAs. During mating, their phallus tears off and they die.

### 1.1.2 Bumblebees (*Bombus* spp.)

Bumblebees are wild bees with a diverse distribution ranging from arctic tundra to tropical forest, but they mainly inhabit cold and temperate climates (Streinzer et al., 2019). Bumblebees contain about 265 species, with 66-70 of them living in Europe (Williams & Jepsen, 2020). The bumblebee species have different lengths of tongue and can tap nectar from flowers of different shapes and depths (Harder, 1983; Heinrich, 1976). It has been shown that bumblebees are suitable for domestication, e.g. *Bombus terrestris*. Because bumblebees produce only small amounts of honey-like substances that cannot be used by humans for honey production, instead the economic focus on rearing them is pollination of crops only (Velthuis & van Doorn, 2006) through which some reared species find almost worldwide distribution.

#### Anatomy and digestive system

Although the external appearance between honey bees and bumblebees differs significantly, e.g. in shape and size, both species belong to the order Hymenoptera and to the family Apidea and are therefore very similar in the general structure of their body and digestive system as described in 1.1.1.

### Castes of bumblebees

Apart from exception of parasitic bumblebees, bumblebees are, like honeybees, eusocial, albeit primitively eusocial (Amsalem et al., 2014). They do have social castes with queens (fertile female), workers (infertile females) and drones (reproduction, at the end of the annual season), dividing labour among queens and workers. But their division of duties is not fixed in terms of life-time and not in the efficient way, as it is in honey bees, but is rather adapted to the present needs of the colony considering the workers to be generalists (Free, 1955; Goulson, 2010).

### Life cycle

Bumblebees form annual colonies (exceptions found in the tropics, where colonies may persist multiannually and are larger in size (Sakagami, 1976)), which distinguishes them from honey bees. Only mated young queens hibernate and found a new colony in spring, using natural cavities as nests. Within the nest the queen creates a supply of pollen (bee bread) and nectar and lays the first approx. 16 eggs. Laying several eggs in one brood cell on a food supply differentiates them from honey bees and is unique to the family Apidae. The queen keeps the eggs at a temperature of about 29 to 32 °C with her body until the first batch of adult workers is matured to take over this task (Vogt, 1986). The larvae are also fed and pupate individually after several moulting steps, as with honey bees. The first workers are usually small, as they were exclusively cared for by the queen and supplies were scarce. As the nest develops, the offspring are better cared for, which allows the following workers to become larger and stronger. In early spring the colonies only consist of a single queen, female workers and immature brood and can grow to a size of 100 - 400 adults (depending on the bee species), which is much smaller than honey bee colonies. In early or late summer (between April and August, depending on the species (Goulson, 2010)) the brood production is changed to males and gynes, i.e. unmated young queens. All individuals from the previous year die, including the old queen (Alford, 1975; Buschinger, 2010; Goulson, 2010). The only survivors that hibernate, as already mentioned at the beginning, are the young queens, i.e. gynes that were mated.

## 1.2 Bee pests and pathogens

The constant presence of the pollinator decline in the news and media creates a great public awareness of the possible dangers and risks, and worrying rates of colony losses have provoked a keen scientific interest in the health of pollinators. Besides anthrophogenic factors such as different use of landscape, pesticides, man-made climate change, also pests and, viral, bacterial and fungal pathogens have a negative impact on bee health (Genersch, 2010; Goulson et al., 2015). Especially pathogens which cause emerging diseases pose a health threat to managed and wild bees worldwide. Following the definition of the WHO "[e]merging diseases are diseases that appear in a population for the first time, or that may have existed previously but are rapidly increasing in incidence or geographic range" (WHO, World Health Organization, http://www.emro.who.int/health-topics/emerging-diseases/index.html, visited May 19, 2022). Among reasons that lead to emerging diseases belong changes in geographic range of pathogens and parasites (Ogden et al., 2017). The main drivers of honey bee colony losses, though not bee declines, are the ectoparasitic mite *Varroa destructor* and the microsporidian parasites *Nosema* spp.

### 1.2.1 Honey bee specific parasite *Varroa destructor*

The most damaging pest of honey bees is the ectoparasitic mite *V. destructor* originating from Southeast Asia (Böcking & Genersch, 2008). It switched from its original host the Eastern honey bee *A. cerana* to the Western honey bee *A. mellifera* at least twice about 70 years ago and was rapidly distributed to all regions with managed honey bees (Traynor et al., 2020) (with a few exceptions like Australia and some remote islands, e.g. Seychelles and Comoros archipelagoes (DAWE, Australian Government: https://www.awe.gov.au/biosecurity-trade/pests-diseases-weeds/bees, visited May 14, 2022; Roberts et al. 2017; Muli et al. 2018). One distinguishes two haplogroups, which were able to parasite *A. mellifera*: the globally spread Korean haplotype (K1) and the Japanese/ Thailand haplotype J1, the latter being abundant in the name-giving countries and on both American continents (Traynor et al., 2020). The mites feed mainly on the fat body of the honey bees (Ramsey et al., 2019). As already mentioned (1.1.1 Anatomy) this organ is of major importance for the honey bee. Damage and restricted function of the fat body leads to negative impact on bee heath status resulting in a weakened energy supply, immune response, hormone regulation and pesticide

detoxification (Arrese & Soulages, 2010), making them vulnerable to pathogens and causing higher winter losses. Of great concern are also viruses vectored and spread by *V. destructor* (Levin et al., 2019; Traynor et al., 2020). Especially DWV is more virulent when passaging the mite before infecting the honey bee (Gisder et al., 2009; Gisder & Genersch, 2021). The complex colony level symptoms caused by DWV and *V. destructor* are called varroosis. At the level of the individual bee, mite-vectored DWV-infections may result in early pupal death or in the emergence of non-viable bees displaying crippled wings and a shortened abdomen or in the emergence of viable bees without visible symptoms but suffering from a DWV-infection of the nervous system and brain resulting in cognitive impairment.

**Life cycle**

*V. destructor* is perfectly adapted to the honey bees' live cycle, such that it even lacks a free living stage. They parasitize honey bees in two particular phases, where they always live bee-linked (Figure 2):

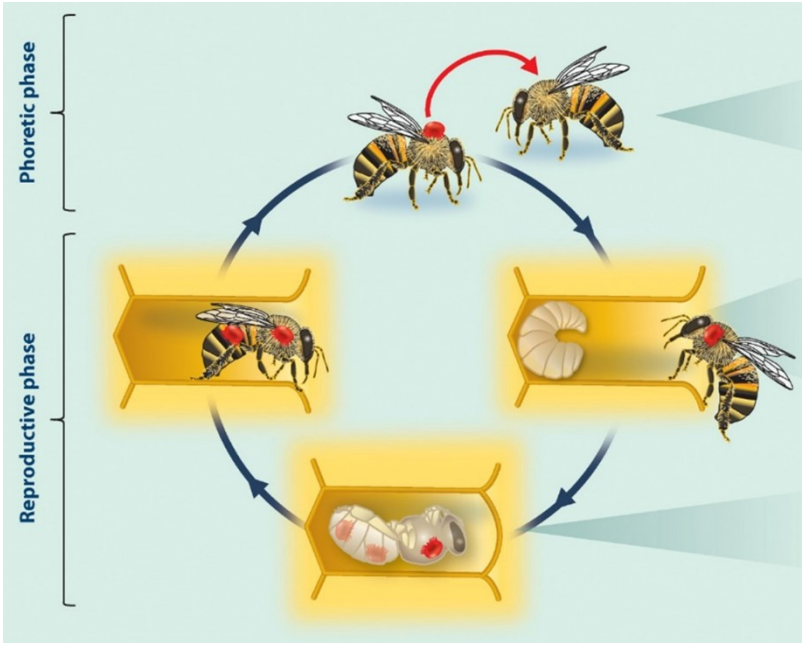



Figure 2: Scheme of the life cycle of the mite *V. destructor*. Picture originally from Nazzi and Conte 2016.

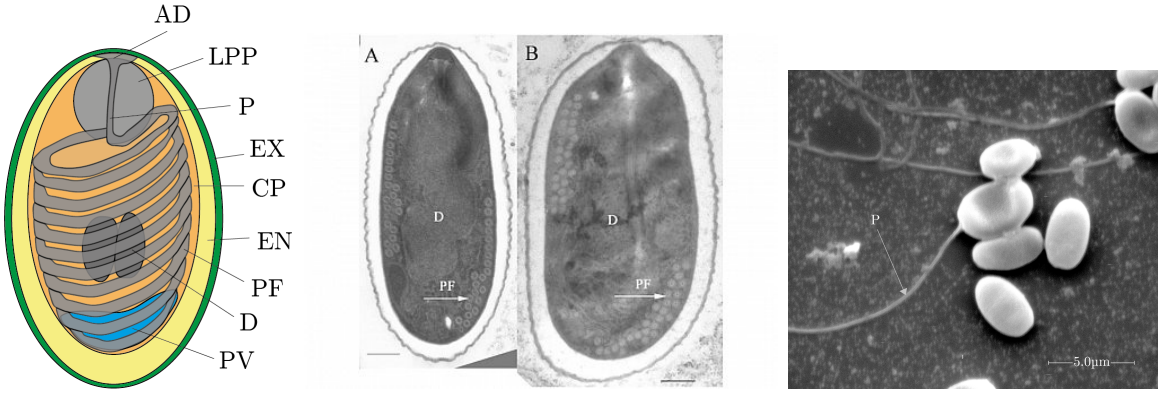
During the phoretic phase/ dispersal phase the adult female mite uses the adult bee as "transport vehicle" and feeds on its fat body. For the reproductive phase, the

female mite lets herself fall into a brood cell of drones or worker bees shortly before cell capping, hence shortly before the beginning of metamorphosis. 70h after capping, while the mite has already parasitized the larvae, her first egg, which is always an unfertilized haploid male, has hatched. The mother mite can lay up to five (in drone brood cells up to six) further fertilized female eggs, of which some mites fully develop (1-2 in workers', up to three in drones' but none in queens' brood cells) and mate with their brother. The copulated female mites leave the brood cell when the bee hatches, starting the phoretic phase where they are able to spread (by foraging or swarming) until they start a new cycle of replication. The male and the virgin female mites die (Rosenkranz et al., 2010).

### 1.2.2 Microsporidia *Nosema* spp.

The major and most widespread microsporidia of *A. mellifera* are *N. apis* and *N. ceranae* (Fries, 2010). *N. neumanni* was recently discovered as a third honey bee pathogenic microsporidium by Chemurot et al. 2017 in Ugandan honey bees and will only be mentioned for the sake of completeness.
According to Tokarev et al. 2020, *"Nosema"* species infecting bees (Anthophila, Hymenoptera) should be redefined within a new genus *"Vairimorpha"*. However, the relevant publication (Tokarev et al., 2020) used molecular data only for reclassification, although it is recommended to use also morphological data for classification of species. According to Figure 1 in Tokarev et al. 2020, the new classification has no advantage but is also very confusing because all morphologies (1 -Sporogony disporoblastic, diplokaryotic (*Nosema*-like); 2 -Sporogony disporoblastic, monokaryotic; 3 -Sporogony octosporoblastic, monokaryotic (*Thelohania*-like)) can be found in the *Nosema* clade and in the *Vairimorpha* clade as well. Furthermore, the chosen journal (Journal of Invertebrate Pathology) is not a taxonomical journal. Therefore, this study is going to stay at the designation of *"Nosema"*, which is still the main form in literature.
Microsporidia spp. are eukaryotic unicellular organisms which belong to the phylum of microsporidia. They are highly specialized fungi (Adl et al., 2005), which proliferate in host cells (obligate intracellular reproduction), which in the case of honey bees are the midgut epithelial cells of their adults. Outside of host cells *Nosema* spp. exist as metabolically inactive endospores. The spores of *N. apis* and *N. ceranae* are similar in size (*N. apis* slightly bigger: 5-7 µm long and 3-4 µm wide; *N. ceranae*: 4-4.8 µm long and 2.1-2.9 µm wide (Aupperle & Genersch, 2016)) and inner structure, which is shown in Figure 3(a) and Figure 3(b) and described in the following.

Briefly, the spore is surrounded by a two-layered wall, called the exospore and endospore, which demarcate the sporoplasm to the outside. One specific characteristic of *Nosema* spp. are the two cell nuclei, which are double connected and presumably polyploidy. Along the membrane of the so-called diplokaryon, the polar filament is coiled. Microsporidia species differ in the number of polar filament coils, e.g. *N. apis* has more than 30 coils, while *N. ceranae* has 18-21 (Chen et al., 2009; Fries et al., 1996). The anchoring disk is located on one narrow side of the spore, in which the




(a) (b) (c)

Figure 3: Spores of *Nosema* spp. as (a) a schematic picture (adopted from Aupperle and Genersch 2016). (b) transmission electron microscope picture of a section of a *N. ceranae* (A) and a *N. apis* (B) spore. Bars = 0.5 µm (picture originally from Fries et al. 2006). (c) a scanning electron microscope picture of several spores with extruded polar filament. Bar = 5 µm. All rights are owned by Genersch, LIB Hohen Neuendorf.
Abbreviations: AD: anchoring disc, LPP: lamellar polaroplast, P: polar filament, EX: exospore, CP: cyto-plasm, EN: endospore, PF: polar filament coils, D: diplokarya (two nuclei), PV: posterior vacuole.

polar filament and a structure called lamellar polaroplast is attached. Furthermore, there is a posterior vacuole in the spore, which creates the pressure for popping out of the polar filament (Han & Weiss, 2017). Oddly, spores lack mitochondria. They only have tiny mitochondrial remnants called mitosomes. Due to that and extreme genomic reduction, there are not able to synthesize ATP on their own. They are strongly dependent on hosts‘ ATP (Aliferis et al., 2012). Close to the plasma membrane they take up ATP via specific nucleotide transport proteins from the host‘s mitochondria (Hacker et al., 2014; Heinz et al., 2014).

**Reproduction cycle**

*Nosema* spp. infection is initiated by ingestion of spores which germinate in the midgut lumen and expel the polar tube (post-germination polar filament, shown in Figure 3(c)). The polar tube pierces an epithelial cell from distance followed by injection of sporoplasm into the host cell (Figure 4 0h). The reproductive circle further comprises

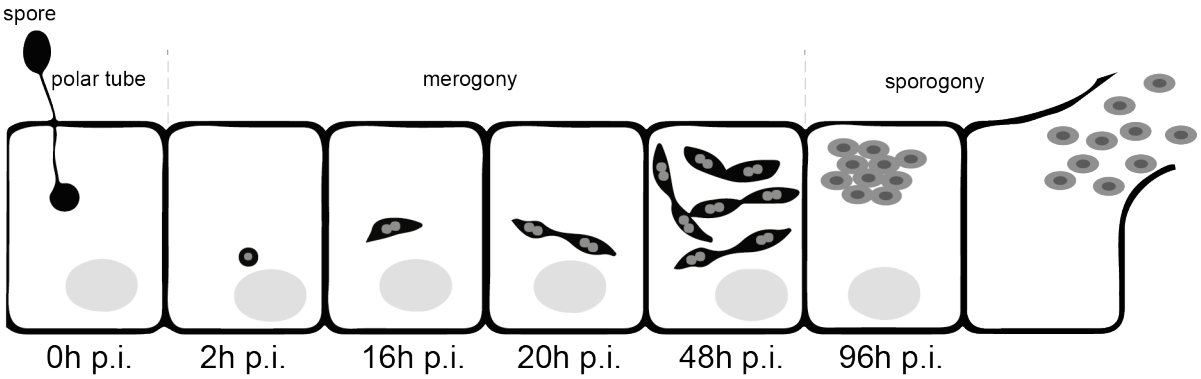



Figure 4: Time line of replication stages of *Nosema ceranae* in honey bee epithelial cells. The injection of the sporoplasm into host cell via polar tube marks time point 0h post infection (p.i.). The merogony–phase comprise four stages and spans the time from 2h to 48h p.i. when sporoplasm is in the host cell as spherical body, forms the spindle-shaped meront, proliferates to a paired meront, and replicates via several rounds of cell division. After 96h p.i. one speaks of sporogony when single round sporonts form and are released as mature spores to the environment (modified from Gisder et al. 2011).

a proliferative phase called merogony and a spore forming phase, the sporogony as illustrated in Figure 4 and described in the following. After invasion, the spherical sporoplasm matures into a meront.

A spindly, double-nucleated meront forms first, becomes a paired meront, which then proliferates. After $\sim$ 48h they separate and develop to oval sporonts. The intracellular life is completed after 96h (Gisder et al., 2011) (Figure 4). Then the mature spores are released into the midgut and contain primary and environmental spores. In case of high proliferation many epithelial cells lyse and further epithelial cells become infected, which restricts functionality of the epithelial layer. Through uncontrolled defecation in the bee comb other worker bees engaged in cleaning activities become infected.

### Clinical picture of *N. apis* and *N. ceranae* infection

*N. apis* has been known to infect the Western honey bee *A. mellifera* for over a century (Zander, 1909) and its pathogenicity is well researched and considered to be of low impact on colony health. *N. apis* infections are known to follow a seasonal pattern with low infections during warm summer and autumn month and increasing incidences from autumn on during the winter month, peaking in spring (Bailey & Ball, 1991; Fries, 1993). Mostly, the infection is covert and infected bees do not show clear clinical symptoms. When the disease nosemosis breaks out, the bees get diarrhoea resulting in characteristic fecal spots on combs and frames (Bailey & Ball, 1991; Fries, 2013). *N. apis* occurs globally, although sharply declining prevalences have been reported in some regions and attributed to the increase in prevalence of the relatively new species *N. ceranae*, that has the potential to replace *N. apis* (Chen et al., 2012; Matthijs et al., 2020). Publications assume a South to North gradient with *N. ceranae* being dominant regarding *N. apis* in Europe which leads to a possible relationship between the climate and *N. ceranae* spread (Chen et al., 2012; Gisder et al., 2010; Klee et al., 2007; Natsopoulou et al., 2015). *N. ceranae* was thought to be specific for *A. cerana* (Fries et al., 1996) but it switched host decades ago and can also infest *A. mellifera* (Higes et al., 2006; Huang et al., 2007). The establishment of a molecular differentiation, e.g. based on 16S rRNA restriction fragment length polymorphisms (RFLP) (Klee et al., 2007) or based on the protein coding gene of DNA-dependent RNA polymerase II largest subunit (RPB1) of a duplex-PCR (Gisder & Genersch, 2013), enabled fast and correct differentiation of both species and clearly demonstrated worldwide distribution as single or co-infection together with *N. apis*. This host switch has contributed to the wide spreading of *N. ceranae*, because *A. mellifera* is globally used as a commercial pollinator (Klee et al., 2007), see also Figure 5.

The impact of *N. ceranae* infections on *A. mellifera* colonies and populations is controversially discussed. In the warmer European countries, especially in Spain, data suggested that *N. ceranae* infection is correlated with honey bee colony losses assuming *N. creanae* to be more virulent than *N. apis* and displacing its congener (Botías et al., 2013; Higes et al., 2008; Higes et al., 2009; Martín-Hernández et al., 2011; Martín-Hernández et al., 2007; Paxton et al., 2007). Furthermore, *N. ceranae* infection has been characterised as asymptomatic and of unseasonal infection prevalence resulting in continuously high infection values throughout the year, which led to the proposal of an alleged new type of nosemosis, the “type C nosemosis” (Higes et al., 2006; Higes


Figure 5: Worldwide distribution of *Nosema* spp. infecting domesticated (white star), wild (black star) or both bees. The review summarizes environmental survey studies, which mainly used PCR as diagnostic method. Next to honey bees and bumblebees also other bees are included. *N. apis*, *N. ceranae* and also other bee *Nosema* spp. are shown (Grupe & Quandt, 2020).

et al., 2010; Martín-Hernández et al., 2007). However, there are also several studies not substantiating a relation between colony losses and *N. ceranae* infection and also questioning increased virulence of *N. ceranae*, asymptomatic course of disease and the displacement of *N. apis* by *N. ceranae* (Fernández et al., 2012; Gisder et al., 2010; Horchler et al., 2018; Stevanovic et al., 2013; Stevanovic et al., 2011).

While microsporidia are described to be rather host specific, the honey bee pathogen *N. ceranae* was reported to be an exception to this rule since it was detected in bumblebees (Fürst et al., 2014; Graystock et al., 2013; Plischuk et al., 2009), solitary bees such as *Osmia* spp. (Ravoet et al., 2014), stingless bees of the genus *Meliponini* (*Tetragonula hockingsi* in Australia (Purkiss & Lach, 2019)), the social wasp *Polybia scutellaris* (*Vespidae*) in south America (Porrini et al., 2017) and greater wax moth *Galleria mellonella* (Özgör, 2021).

Cases of *N. ceranae* infections in bumblebees broached the issue of "interspecies-transmission" of *N. ceranae* as an emergent pathogen in bumblebee *Bombus terrestris* (Graystock et al., 2013; Plischuk et al., 2009). Interestingly, the common feature of these studies was that they used PCR-based protocols to detect *N. ceranae*, but did not look for or find newly produced spores, which would have indicated true infection. Hence, the question of whether *N. ceranae* is a promiscuous pathogen truly infecting many other hymenopteran species was not finally answered by these studies.

# 2 Results

As outlined above, *N. ceranae* is a relatively new pathogen for the Western honey bee which switched host from *A. ceranae* to *A. mellifera* only some decades ago. Following this host switch, *N. ceranae* has also been reported to have switched again, now from *A. mellifera* to several other hymenopteran species. Although there are numerous studies on the pathobiology of *N. ceranae*, it is still not as well studied as its congener *N. apis*. Therefore, the aim of this thesis was to answer some of the open questions regarding *N. ceranae* spread, seasonality, dominance, virulence, and host range:
In Gisder et al. 2017 (attached in 2.1) we addressed the questions (i) of *N. ceranae* prevalence and spreading within the honey bee population in Northeast Germany, (ii) of seasonality of *N. ceranae* infections, and (iii) of the dominance of *N. ceranae* over *N. apis* resulting in the displacement of *N. apis*. For this study we used data originating from a long-term monitoring project on honey bee health which started in spring 2005 and is still ongoing. About 23 apiaries located in Northeast Germany participate with 10 colonies each in this monitoring project. Between 2005 and 2016, the prevalence of *N. apis*, *N. ceranae* and co-infections was examined each spring and autumn and colony losses especially over winter were recorded. Our comprehensive statistical analysis of this data revealed (i) a clear seasonality of *N. ceranae* infection prevalence, with higher infection rates in spring than in in autumn; (ii) a significant increase in *N. ceranae* prevalence in spring of about 5% per year and in autumn of about 15% per year during the study period; but (iii) no displacement of *N. apis* by *N. ceranae*. Explanations for the success of *N. ceranae* during summer were provided by cell culture experiments which showed that with increasing incubation temperature, infected cells produced significantly more *N. ceranae* spores than *N. apis* spores suggesting a higher biotic potential of *N. ceranae* during summer compared to *N. apis*.
In Schüler et al. 2022 (attached in 2.2) we again analysed the data provided by the long-term monitoring project on honey bee health in Northeast Germany, but this time covered the period between 2005 and 2020. We addressed the question of virulence at colony level by statistically analysing the data on colony mortality and on *Nosema* spp.-infection, *Nosema* spp. spore load, and *V. destructor* infestation level in autumn.

By using classification tree analysis, contingency table analysis, and determining Cohen's $\omega$, we demonstrated that the majority of colony losses over winter are due to the infestation with the ectoparasitic mite *V. destructor*. Only in the few colonies with no or low mite infestation, *N. ceranae* infections could become a problem. Hence, virulence of *N. ceranae* at colony level is low and is of no or little biological relevance. Furthermore, the question whether *N. ceranae* is related to elevated honey bee winter losses is clarified.

In Gisder et al. 2020 (attached in 2.3) we addressed the question of *N. ceranae* host range by controlled laboratory exposure experiments using *B. terrestris* as proxy for wild bees and *A. mellifera* as control. We analyzed the infection status of the experimentally infected bees (honey bees and bumblebees) by microscopic analysis of squash preparations, PCR-detection protocols, histology of Giemsa-stained tissue sections, and species-specific fluorescence in situ hybridization (FISH). We exposed bumblebees of different ages to increasing doses of *N. ceranae* spores, but never observed true infection of *B. terrestris* with *N. ceranae* suggesting that *N. ceranae* is not able to infect *B. terrestris* and, hence, that *B. terrestris* is not a host for this honey bee specific microsporidium. We followed the fate of the ingested spores in the bumblebee alimentary canal and present the rapid gastro-intestinal passage in bumblebees as a possible explanation for the failure of ingested *N. ceranae* spores to initiate an infection in bumblebees.

## 2.1 Long-Term Temporal Trends of *Nosema* spp. Infection Prevalence in Northeast Germany: Continuous Spread of *Nosema ceranae*, an Emerging Pathogen of Honey Bees (*Apis mellifera*), but No General Replacement of *Nosema apis*

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My contribution to this publication was mainly the curation of the data and the conversion of the huge and complex data set to make it suitable for statistical analysis followed by the statistical analysis of the data and visualization of the results (Fig. 1, 3, 4, 5B-D, 6B-D). I worked on the content revision of the manuscript and approved the final version.

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The Western honey bee (*Apis mellifera*) is widely used as commercial pollinator in worldwide agriculture and, therefore, plays an important role in global food security. Among the parasites and pathogens threatening health and survival of honey bees are two species of microsporidia, *Nosema apis* and *Nosema ceranae*. *Nosema ceranae* is considered an emerging pathogen of the Western honey bee. Reports on the spread of *N. ceranae* suggested that this presumably highly virulent species is replacing its more benign congener *N. apis* in the global *A. mellifera* population. We here present a 12 year longitudinal cohort study on the prevalence of *N. apis* and *N. ceranae* in Northeast Germany. Between 2005 and 2016, a cohort of about 230 honey bee colonies originating from 23 apiaries was sampled twice a year (spring and autumn) resulting in a total of 5,600 bee samples which were subjected to microscopic and molecular analysis for determining the presence of infections with *N. apis* or/and *N. ceranae*. Throughout the entire study period, both *N. apis*- and *N. ceranae*-infections could be diagnosed within the cohort. Logistic regression analysis of the prevalence data demonstrated a significant increase of *N. ceranae*-infections over the last 12 years, both in autumn (reflecting the development during the summer) and in spring (reflecting the development over winter) samples. Cell culture experiments confirmed that *N. ceranae* has a higher proliferative potential than *N. apis* at 27° and 33°C potentially explaining the increase in *N. ceranae* prevalence during summer. In autumn, characterized by generally low infection prevalence, this increase was accompanied by a significant decrease in *N. apis*-infection prevalence. In contrast, in spring, the season with a higher prevalence of infection, no

significant decrease of *N. apis* infections despite a significant increase in *N. ceranae* infections could be observed. Therefore, our data do not support a general advantage of *N. ceranae* over *N. apis* and an overall replacement of *N. apis* by *N. ceranae* in the studied honey bee population.

**Keywords: honey bee, *Apis mellifera*, *Nosema* spp., epidemiology, replacement**

## INTRODUCTION

The Western honey bee *Apis mellifera* is a valuable generalist pollinator for many flowering plants in both natural and agricultural ecosystems. In agriculture, commercial pollination of crop plants, that depend on insect pollination for fruit set and seed production, is provided mostly by managed *A. mellifera* colonies which can, therefore, be regarded as productive livestock. The cultivation of pollinator-dependent crops is expanding all over the world; hence, there is an increasing demand for insect pollination in worldwide agriculture (Aizen et al., 2008, 2009; Aizen and Harder, 2009). Although, this demand is partially met by a globally increasing number of managed honey bee colonies (Aizen et al., 2008, 2009; Moritz and Erler, 2016), increasing problems with honey bee health resulting in severe honey bee colony losses pose a serious threat to human food security. Research of the last decade has identified a multitude of factors like pathogens, pesticides, and abiotic stressors being associated with unusually high and inexplicable losses of honey bee colonies (Genersch, 2010; Ratnieks and Carreck, 2010; Cornman et al., 2012; Pettis et al., 2013; Goulson et al., 2015). Among the pathogens studied and discussed in this context are two microsporidian parasites, *Nosema apis* (*N. apis*) and *N. ceranae,* (Cox-Foster et al., 2007; Higes et al., 2008; Genersch, 2010) which infect adult honey bees (Bailey, 1955).

Microsporidia are highly specialized, spore-forming fungi which are optimally adapted to an obligate intracellular parasitic life style (Keeling and Fast, 2002). Outside of host cells, microsporidia exist as metabolically inactive, infective spores. For *N. apis* and *N. ceranae*, the infection process starts with the ingestion of infective spores by an adult honey bee. The spores germinate in the midgut thereby extruding the polar tube. If the polar tube pierces a host cell, the sporoplasm is injected into the cell through the polar tube (Bigliardi and Sacchi, 2001; Franzen, 2005). Following the injection of the sporoplasm, it takes about 96 h until the first environmental spores are produced by an infected cell (Gisder et al., 2011). The spores are released into the gut lumen through cell lysis and leave the body of the infected host by defecation (Bailey, 1955; Bailey and Ball, 1991). Heavy *Nosema* spp.-infections of adult honey bees may result in dysentery (Bailey, 1967). Adult bees suffering from diarrhea will show abnormal defecation behavior, i.e., will defecate inside the hive, resulting in fecal spots on combs and frames. Nest mates cleaning these spots will ingest *Nosema* spp. spores and become infected (Bailey and Ball, 1991). Infections with *Nosema* spp. are widespread in honey bee populations. Most infected honey bees do not develop nosemosis and do not show any obvious symptoms like dysentery but may have an increased foraging or flight activity (Woyciechowski and Kozlowski, 1998; Dussaubat et al., 2013) despite impaired orientation and homing skills (Kralj and Fuchs, 2010; Wolf et al., 2014) and may have a suppressed immune system (Antunez et al., 2009; Chaimanee et al., 2012), as well as a reduced life span (Wang and Moeller, 1970; Malone and Giacon, 1996; Fries, 2010).

*Nosema apis*-infections in honey bees have been studied intensively over the last 100 years and there is little debate on the rather low impact of this parasite on *A. mellifera* colonies (Bailey and Ball, 1991). However, the impact of *N. ceranae*-infections on colony health and survival is still controversially discussed (Higes et al., 2008; Genersch et al., 2010; Gisder et al., 2010; Guzman-Novoa et al., 2011; Stevanovic et al., 2011; Fernández et al., 2012). The emerging picture is that *N. ceranae* might cause colony death in warmer climates like Southern Europe (Higes et al., 2007, 2008, 2009; Martin-Hernandez et al., 2007; Botías et al., 2013; Cepero et al., 2014) whereas colony losses in Northern Europe or the Americas could not be associated with *N. ceranae* so far (Invernizzi et al., 2009; Genersch et al., 2010; Gisder et al., 2010; Williams et al., 2010; Guzman-Novoa et al., 2011) suggesting a climatic influence on *N. ceranae* virulence (Gisder et al., 2010) or differences in *N. ceranae* susceptibility between regionally predominating *A. mellifera* subspecies (Fontbonne et al., 2013; Huang et al., 2015).

Initially it was thought that *N. apis* is specific for the Western honey bee *A. mellifera* (Zander, 1909), while its congener *N. ceranae* was described as a microsporidian parasite of the Eastern honey bee *A. cerana* (Fries et al., 1996), a native of South- and Southeast Asia. Although, experimental infection showed from the very beginning that *N. ceranae* can also successfully infect *A. mellifera* (Fries, 1997), it took nearly a decade until the first natural infections of *A. mellifera* colonies with *N. ceranae* were reported (Higes et al., 2006; Huang et al., 2007). It soon became evident that *N. ceranae* was not only much more widespread than expected in the global *A. mellifera* populations but that is was even the predominant species in many regions (Klee et al., 2007; Chen et al., 2008; Williams et al., 2008; Invernizzi et al., 2009; Chen and Huang, 2010; Yoshiyama and Kimura, 2011; Copley et al., 2012). Based on this epidemiological evidence it was suggested that *N. ceranae* is replacing *N. apis* in the honey bee populations worldwide. This process is thought to be driven by an asymmetric within-host competition between *N. apis* and *N. ceranae* favoring the spread of *N. ceranae* (Williams et al., 2014; Natsopoulou et al., 2015) although not all studies observed interspecific competition between *N. apis* and its congener *N. ceranae* (Forsgren and Fries, 2010; Milbrath et al., 2015).

However, a pan-European study on the prevalence of *N. apis* and *N. ceranae* reported that in South-European countries, such as Italy and Greece, *N. ceranae* had indeed practically replaced *N. apis* while this was not observed in Northern Europe (Ireland, Sweden, Norway, and Germany) (Klee et al., 2007). These data pointed to climatic factors differentially influencing

assertiveness, establishment, spread, and, hence, prevalence of *N. apis* and *N. ceranae*. Experimental evidence exists showing that *N. ceranae* spores, but not *N. apis* spores, nearly lose their ability to germinate and, hence, their infectivity when exposed to temperatures close to or below freezing (Fenoy et al., 2009; Fries, 2010; Gisder et al., 2010). In addition, experimental infection of adult bees showed proliferation of *N. ceranae*—but not of *N. apis*—to be unaffected by temperatures above 33°C (Martin-Hernandez et al., 2009). These data strongly argue for an advantage of *N. ceranae* over *N. apis* in warmer climates. In contrast, the cold-sensitivity of *N. ceranae* spores might slow down the replacement process in colder climates (Gisder et al., 2010), a hypothesis that could recently be substantiated by mathematical modeling of the replacement process when taking into account the parameters warmer and colder climate (Natsopoulou et al., 2015). However, long term epidemiological data on *Nosema* spp. prevalence allowing the observation of the spread of the emerging pathogen *N. ceranae* and evaluating the proposed process of replacement of *N. apis* by *N. ceranae* in a given honey bee population have been lacking so far. To fill this gap, we here present our results of a 12 year cohort study on the prevalence of *N. apis* and *N. ceranae* in Northeast Germany conducted on a cohort of about 230 honey bee colonies. The duration of the study, and the size of the cohort enabled us to statistically analyse the long term temporal trends in prevalence of *N. apis*- and *N. ceranae*-infections in the study area. We also show data from laboratory experiments substantiating our epidemiological data. We provide evidence that the continuous spread of *N. ceranae* and continuously increasing levels of *N. ceranae*-infection prevalence at population level not necessarily result in the replacement of *N. apis*.

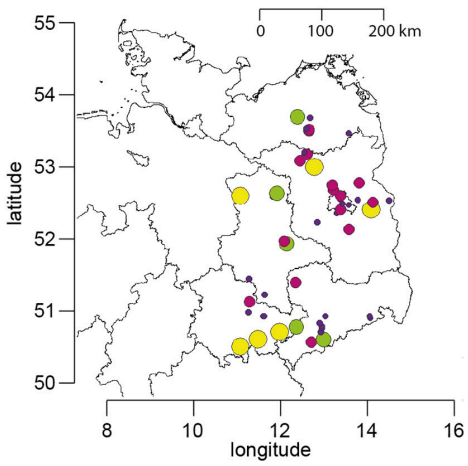


**FIGURE 1 |** Map of Northeast Germany showing the location of the apiaries which participated in the study. The size and color of the circles represent the number of years for which data are available for each apiary (yellow, 12 years; green, 9–11 years; purple, 5–8 years; blue, 1–4 years).

## MATERIAL AND METHODS

### Bee Samples, Field Survey and Molecular Differentiation of *N. apis* and *N. ceranae*

The data set on *Nosema* spp. prevalence comprises data from spring 2005 to autumn 2016, which were collected in the course of a 5 year longitudinal cohort-study on *Nosema* spp. epidemiology (Gisder et al., 2010) and of the still ongoing "German Bee Monitoring Project" (Genersch et al., 2010). About 23 apiaries located in Northeast-Germany (**Figure 1**) participated in the projects with 10 colonies ("monitoring colonies") each. Monitoring colonies that collapsed during the study were replaced by colonies from the same apiary, if available by a nucleus colony made from the collapsed colony in the previous year. This procedure ensured that each apiary always contributed 10 monitoring colonies throughout the study period. Due to the long duration of the study, some fluctuation of participating apiaries could not be avoided. However, nearly half of the apiaries (11 of ∼23) participated for more than 9 years and six of them even for the entire duration of the study, i.e., 12 years; at least 20 bee keepers provided samples over a time period of consecutive 5–11 years (**Figure 1**). When an apiary dropped out, a similar apiary in terms of size, bee race, landscape, region, and history of losses and diseases was chosen as replacement and included in the study as soon as possible. This resulted in an annual mean of 22.67 ± 1.72 (mean ± *SD*) apiaries participating in spring and 24.0 ± 2.83 (mean ± *SD*) apiaries participating in autumn. All monitoring colonies were sampled twice a year, in spring and in autumn, resulting in a total of 5,600 honeybee samples collected and analyzed from the participating apiaries over the 12 year study period (**Table 1**).

Sampling of bees as well as diagnosis of *N. apis* and *N. ceranae* were performed essentially as already described (Gisder et al., 2010). Briefly, from each apiary, a group of 10 bee colonies [annual mean: 10 ± 0.31 (mean ± *SD*) colonies in spring and 10.01 ± 0.14 (mean ± *SD*) colonies in autumn] was randomly selected at the beginning of the study or when the beekeeper entered the study and designated "monitoring colonies." From these colonies, bee samples were collected in spring and autumn each year and were stored at −20°C until analysis. Spring samples collected end of March/beginning of April consisted of dead bees fallen onto the bottom board during the winter season (representing the bees that died over winter) to enable sampling of colonies that collapsed during the winter season (October to March) as well as of surviving colonies. Autumn samples collected in late September/beginning of October consisted of live in-hive bees taken from a super above the queen excluder thus ensuring that only the oldest bees (representing the most frequently infected bees) were sampled (Fries et al., 2013). Diagnosis of *Nosema* spp. infections was performed by microscopic examination of 20 homogenized bee abdomens according to the "Manual of Standards for Diagnostics and Vaccines" published by the Office International des Epizooties (OIE), the World Organization for Animal Health (Anonymous, 2008). The moderate sample

**TABLE 1 |** Prevalence of colonies infected with *N. apis* only (*N. apis*) or *N. ceranae* only (*N. ceranae*) or with *N. apis* and *N. ceranae* (co-infection) from spring 2005 to autumn 2016.

| | | Total number of analyzed colonies | Infected Colonies [infection categories] | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | *N. apis* | | *N. ceranae* | | co-infection | |
| | | | *n* | % | *n* | % | *n* | % |
| Spring | 2005 | 220 | 37 | 16.8 | 9 | 4.1 | 6 | 2.7 |
| Autumn | 2005 | 237 | 19 | 8.0 | 10 | 4.2 | 1 | 0.4 |
| Spring | 2006 | 238 | 43 | 18.1 | 10 | 4.2 | 14 | 5.9 |
| Autumn | 2006 | 226 | 15 | 6.6 | 3 | 1.3 | 2 | 0.9 |
| Spring | 2007 | 228 | 7 | 3.1 | 34 | 14.9 | 10 | 4.4 |
| Autumn | 2007 | 219 | 10 | 4.6 | 4 | 1.8 | 1 | 0.5 |
| Spring | 2008 | 209 | 35 | 16.8 | 18 | 8.6 | 21 | 10.1 |
| Autumn | 2008 | 210 | 6 | 2.9 | 5 | 2.4 | 0 | 0.0 |
| Spring | 2009 | 210 | 33 | 15.7 | 23 | 11.0 | 12 | 5.7 |
| Autumn | 2009 | 180 | 9 | 5.0 | 7 | 3.9 | 0 | 0.0 |
| Spring | 2010 | 247 | 27 | 10.9 | 25 | 10.1 | 4 | 1.6 |
| Autumn | 2010 | 250 | 10 | 4.0 | 16 | 6.4 | 0 | 0.0 |
| Spring | 2011 | 230 | 42 | 18.3 | 25 | 10.9 | 16 | 7.0 |
| Autumn | 2011 | 255 | 7 | 2.8 | 7 | 2.8 | 5 | 2.0 |
| Spring | 2012 | 252 | 24 | 9.5 | 33 | 13.1 | 23 | 9.1 |
| Autumn | 2012 | 278 | 6 | 2.2 | 19 | 6.8 | 0 | 0.0 |
| Spring | 2013 | 233 | 15 | 6.4 | 30 | 12.9 | 12 | 5.2 |
| Autumn | 2013 | 257 | 17 | 6.6 | 21 | 8.2 | 4 | 1.6 |
| Spring | 2014 | 224 | 41 | 18.3 | 33 | 14.7 | 7 | 3.1 |
| Autumn | 2014 | 261 | 4 | 1.5 | 8 | 3.1 | 0 | 0.0 |
| Spring | 2015 | 198 | 25 | 12.6 | 15 | 7.6 | 7 | 3.5 |
| Autumn | 2015 | 250 | 5 | 2.0 | 3 | 1.2 | 0 | 0.0 |
| Spring | 2016 | 230 | 43 | 18.7 | 21 | 9.1 | 4 | 1.7 |
| Autumn | 2016 | 258 | 8 | 3.1 | 27 | 10.5 | 0 | 0.0 |

*Given are the total number of analyzed colonies per year and season as well as the numbers (n) and proportions (%) of colonies within each infection category.*

size is adequate because the experimental unit is the colony (Doull and Cellier, 1961; Doull, 1965). Infection status of the colonies represents detectable levels of infection above 15% with 96% probability of detection (Fries et al., 1984, 2013; Pirk et al., 2013) which can be considered biologically relevant (Higes et al., 2008). For molecular species differentiation, *Nosema* spp.-positive homogenates were processed and analyzed via PCR-RFLP (restriction fragment length polymorphism) as previously described (Gisder et al., 2010). Results were further verified by re-analyzing randomly selected samples via a recently developed differentiation protocol (Gisder and Genersch, 2013) which is based on the detection of species-specific sequence differences in the highly conserved gene coding for the DNA-dependent RNA polymerase II largest subunit. Based on the diagnostic results, four infection categories were defined: Microscopic analysis resulted in the category "*Nosema* spp." while molecular differentiation allowed for the categories "*N. apis*" (single infection), "*N. ceranae*" (single infection), and "co-infection" (infection with both *N. ceranae* and *N. apis*) (**Table 1**).

## Purification of *Nosema* spp. Spores for *In Vitro*-Infection

Honey bee colonies of the apiary of the Institute for Bee Research were screened for *Nosema* spp.-infections by microscopic analysis of 20 randomly collected adult bees (see above and Anonymous, 2008). *Nosema* spp.-positive samples were molecularly differentiated as previously described (Gisder and Genersch, 2013) to identify samples either containing only *N. ceranae* or only *N. apis* spores. Purification of *N. apis* or *N. cerane* spores was exclusively performed with freshly sampled bees, because freezing or long-term storage affect spore viability and infection rate (Fenoy et al., 2009; Fries, 2010; Gisder et al., 2010). Midguts were carefully isolated from individual bees by using fresh forceps for each bee. Twenty midguts were pooled in 1.5 ml reaction tubes and spore purification was performed as already described (Gisder et al., 2010).

Viability of the purified spores was checked via *in vitro*-germination. To this end, an aliquot of freshly isolated spores was air-dried onto glass slides for 30 min at room temperature. Germination was triggered by adding 20 µl of 0.1 M sucrose solution buffer directly to the dried spores. Germination process was analyzed under an inverse microscope (VWR, Darmstadt, Germany) at 400x magnification with phase contrast. *Nosema* spp. spores were counted in a hemocytometer (Neubauer-improved, VWR, Darmstadt, Germany) under an inverse microscope (VWR, Darmstadt, Germany) at 100x magnification. Only those spore preparations that were able to germinate under *in vitro* conditions were used for cell culture experiments.

## *In vitro*-Infection of Cultured IPL-LD-65Y Cells

The insect cell line IPL-LD-65Y derived from the gypsy moth *Lymantria dispar* was obtained from the *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DSMZ, Braunschweig, Germany) and maintained for routine culture as given in the accompanying data sheet. For *in vitro*-infection of cultured IPL-LD-65Y-cells, aliquots of about $5 \times 10^7$ spores, purified as described above, were dried in 1.5 ml reaction tubes (Eppendorf, Hamburg, Germany) in a vacuum concentrator (Eppendorf, Hamburg, Germany) for 30 min at 30°C. Subsequently, infection of IPL-LD-65Y cells with germinating spores was performed as previously described (Gisder et al., 2011). Briefly, IPL-LD-65Y cells were infected with freshly isolated *N. apis* or *N. ceranae* spores with a multiplicity of infection (MOI) of 20. Infected cells (100 µl with $2.5 \times 10^5$ cells/ml) were seeded in the cavities of six 96-well microtiter plates. *N. apis*- and *N. ceranae*-infected cells were incubated at 21°, 27°, or 33°C. Infected cells were centrifuged on glass slides at the time points 24, 32, 48, 72, and 96 h post initial infection and were subsequently Giemsa-stained as described (Gisder et al., 2011). The number of meronts, sporonts, and mature spores of *N. apis* or *N. ceranae* was counted under an inverse microscope Eclipse Ti-E (Nikon Instruments, Düsseldorf, Germany) at 600x magnification in 10 individual cells for each time point as well as for each temperature and expressed as mean ± *SD*.

## Statistical Analysis

For statistically analyzing the seasonality of *Nosema* infections, spring vs. autumn, the Wilcoxon signed rank test was used because the proportions of infected colonies (**Table 1**) were not normally distributed. In addition, the Spearman rank correlation was determined with R (version 3.2.5, R Development Core Team, 2016) to analyse the relationship between infection categories. The Spearman correlation coefficient determined the strength of the monotonic relationship between season and infection prevalence with effect sizes between 0.10 and 0.29 representing weak correlations, coefficients between 0.30 and 0.49 representing medium correlations, and coefficients of 0.50 or above representing strong correlations.

For each time point, the expected rate of co-infections ($E_{co-inf}$) was calculated as the product of the observed rates of single infections with either *N. apis* ($R_{apis}$) or *N. ceranae* ($R_{ceranae}$): $E_{co-inf} = R_{apis} * R_{ceranae}$. Subsequently, the differences between the observed and expected rates of co-infections were calculated for each time point. Because those differences were normally distributed, a one sample *t*-test was used to check if these differences were significantly different to zero.

The statistical analysis of temporal trends was performed using RStudio (version 0.99.489) based on R using version 3.2.5. For visualizing infection prevalence data, dotplots were plotted with R, separately for spring and autumn. Generalized linear models (GLM) were fitted with lme4 (Linear Mixed-Effects Models, version 1.1-12) (Bates et al., 2015) for exploring the data set and visualizing the relationship between the dependent variables (*Nosema* spp.) and the independent variables (year). For statistical analysis of *N. apis* and *N. ceranae* prevalence over the 12 year study period we used mixed-effect binary logistic regressions analysis defining year as fixed factor and apiary as random factor to take into account the lack of independence of data within each apiary. Even after 12 years of sampling, the amount of data is still not sufficient to define colony as random factor to fully acknowledge relative data dependence. The sampling consisted of about 230 individual colonies per season, stratified within apiaries, and the prevalence of *N. apis*-, *N. ceranae*-, or co-infections at the individual level were analyzed with defining "0" if absent or "1" if present in each colony. Odds ratios (ORs) and 95% confidence intervals [CIs] were used to assess the strength of the associations.

For statistical analysis of the counted number of different developmental stages of *Nosema* spp. in infected IPL-LD-65Y cells, individual student's *t*-tests for each time point were performed followed by Benjamini-Hochberg correction (Benjamini and Hochberg, 1995). A $p < 0.05$ was considered significant for the statistical tests.

# RESULTS

## Prevalence and Seasonality of *Nosema* spp.-Infections

The huge data set on *Nosema* spp.-infection prevalence in Northeast Germany, which was generated during the 12 year longitudinal cohort study, provided a unique opportunity for a comprehensive analysis of the spread and success of *Nosema* spp., and especially of *N. ceranae*, in a restricted honey bee population. We first analyzed the seasonality of *Nosema* spp.-infections based on classical microscopic diagnosis without molecular species differentiation. The data revealed a clear and expected (Bailey and Ball, 1991) seasonality of *Nosema* spp.-infections for the whole duration of the study period with spring values being always higher than the autumn values of the same year and autumn values being always lower than the spring values of the following year (**Figure 2A**).

Molecular species differentiation of all *Nosema* spp.-positive samples enabled analysing the seasonality of *N. apis*-, *N. ceranae*-, and co-infections (**Table 1**). The same seasonality as already observed for *Nosema* spp.-infections was also evident for *N. apis*-infections over the entire study duration despite for the time point "spring 2007" when less colonies where found infected with *N. apis* than in the preceding autumn 2006 and the following autumn 2007 (**Figure 2B**). With this exception for "spring 2007," when only 3.1% of the colonies carried detectable *N. apis*-infections, the proportion of *N. apis*-infected colonies varied between 6.4% (spring 2013), and 18.7% (spring 2016). In autumn, the prevalence of *N. apis*-infected colonies ranged between 1.5% (autumn 2014) and 8.0% (autumn 2005).

For *N. ceranae*-infections, the described seasonality with higher prevalence in spring than in the following autumn and lower prevalence in autumn than in spring next year could be observed from autumn 2006 onward until spring 2016, whereas between spring and autumn 2016 the prevalence of *N. ceranae*-infections did not decrease as expected but instead further increased (from 9.1 to 10.5%; **Figure 2B**). Spring prevalence from 2007 to 2016 varied for *N. ceranae*-infections between 7.6% (spring 2015) and 14.9% (spring 2007), while autumn prevalence ranged between 1.2% (autumn 2015) and 8.2% (autumn 2013).

The prevalence of colonies co-infected with *N. apis* and *N. ceranae* showed the same seasonal pattern fluctuating between spring (higher prevalence) and autumn (lower to no prevalence). Values for co-infection prevalence ranged between 1.6% (spring 2010) and 10.0% (spring 2008) in spring and between 0.0% (autumn 2008, 2009, 2010, 2012, 2014, 2015) and 2.0% (autumn 2011) in autumn (**Figure 2B**).

Statistical analysis of the seasonality of *Nosema* spp.-, *N. apis*-, *N. ceranae*-, and co-infections using a Mann-Whitney test confirmed the above given, rather descriptive evaluation (for all infection categories, $p < 0.01$). Spearman correlation analysis further substantiated this finding (**Figure 3**). A strong negative correlation (coefficient values between −0.69 and −0.87) was found between season and all infection categories indicating that in each year and for all four infection categories (*Nosema* spp.-, *N. apis*-, *N. ceranae*-, co-infection) the infection prevalence decreased significantly from spring to autumn (for all infection categories: $p < 0.01$). Medium to strong positive correlations (coefficient values between 0.44 and 0.85) were found between the infection categories implying that all infection categories followed the same prevalence trend. For example, high infection prevalence for *N. apis* correlated with high infection prevalence for *N. ceranae*- or co-infections. This correlation was significant for all infection categories ($p < 0.05$).

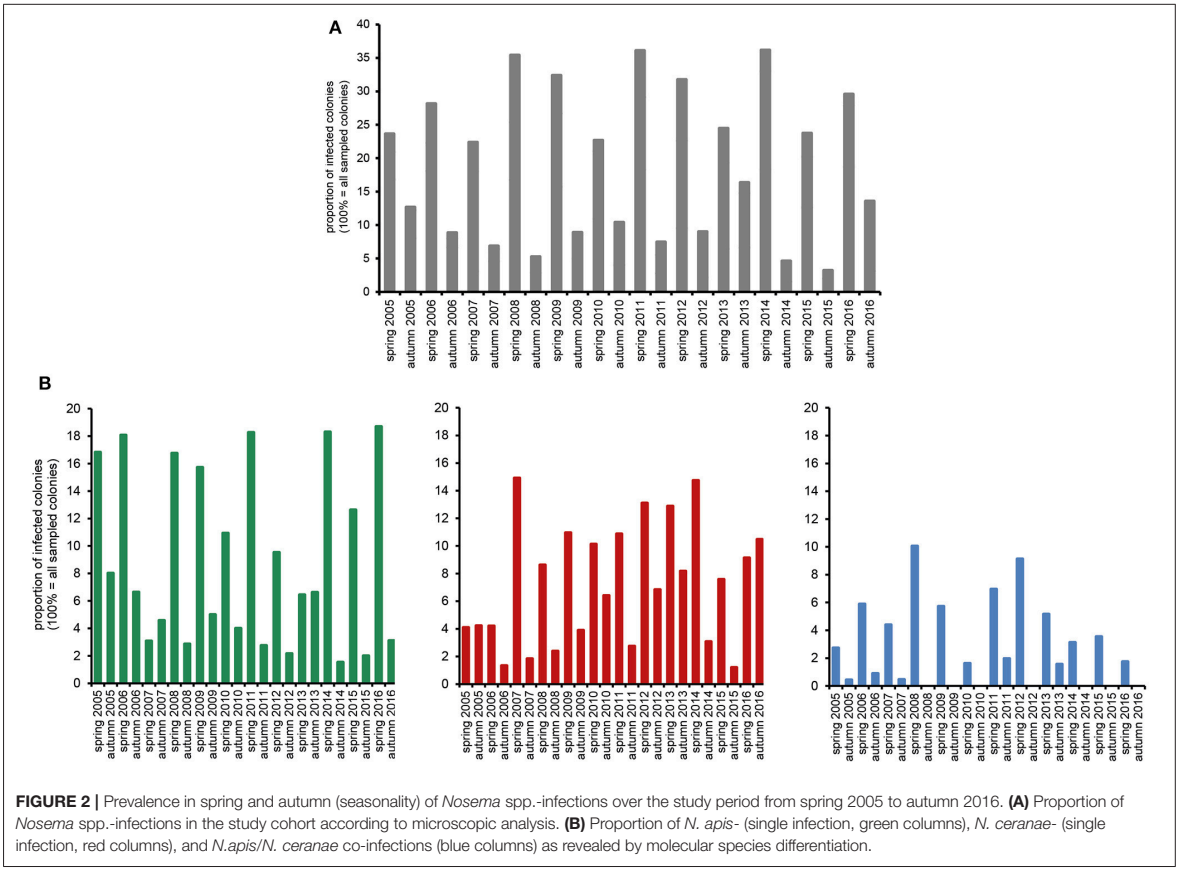


**FIGURE 2 |** Prevalence in spring and autumn (seasonality) of *Nosema* spp.-infections over the study period from spring 2005 to autumn 2016. **(A)** Proportion of *Nosema* spp.-infections in the study cohort according to microscopic analysis. **(B)** Proportion of *N. apis*- (single infection, green columns), *N. ceranae*- (single infection, red columns), and *N.apis*/*N. ceranae* co-infections (blue columns) as revealed by molecular species differentiation.

An interesting question in regard to co-infections was, whether or not the observed prevalence of co-infections in spring was congruent with the expected prevalence. To answer this question, we first calculated the rate of expected co-infections for each year from the rate of observed *N. apis*- and *N. ceranae*-infections in this season. Comparing these values with the observed frequency of co-infections revealed that over the entire study period, the observed prevalence of co-infections was always significantly {one sample *t*-test; $M = 0.037$, [0.0195, 0.0546], $t_{(23)} = 4.6389$, $p < 0.01$} higher than expected when assuming that the occurrence of co-infections was only influenced by the prevalence of single infections (**Figure 4**).

## Temporal pattern of *Nosema* spp. Prevalence

For evaluating spread and assertiveness of the emerging honey bee pathogen *N. ceranae*, we analyzed the temporal patterns of *N. ceranae*-, *N. apis*-, and co-infections by plotting and statistically analysing the respective values separately for the spring (**Figure 5**) and autumn (**Figure 6**) seasons between 2005 and 2016. While the patterns for *N. apis*- and co-infections in spring did not show a consistent trend, the pattern for *N. ceranae*-infection prevalence suggested a continuously increasing trend over the years (**Figure 5A**). Generalized linear models (GLM) of the prevalence data confirmed this interpretation (**Figures 5B–D**). Logistic regression analysis (**Table 2**) demonstrated that the continuous increase in spring prevalence of *N. ceranae*-infections observed over the entire 12 year study period, i.e., between 2005 and 2016, was on average about 5% per year (Odd Ratio: 1.05 [1.01, 1.1]) and was significant (GLM, Likelihood Ratio test of the model, $p = 0.02$) (**Figure 5B**). This increase, however, was not accompanied by any significant (GLM, Likelihood Ratio test of the model, $p = 0.95$) change in the spring prevalence of *N. apis*-infections (Odd Ratio: 1.0 [0.96, 1.04]) (**Figure 5C**). Likewise, no significant trends (GLM, Likelihood Ratio test of the model, $p = 0.17$) were observed for co-infections in spring (Odd Ratio: 0.96 [0.9, 1.02]) (**Figure 5D**).

The dotplot of autumn prevalence of *N. ceranae*-, *N. apis*-, and co-infections (**Figure 6A**) showed a different pattern with an increasing trend for *N. ceranae*- being accompanied by a decreasing trend for *N. apis*-infections. This finding could be substantiated by GLM-analysis and Likelihood Ratio tests of the models (**Table 2**, **Figures 6B–D**). In autumn, the prevalence of

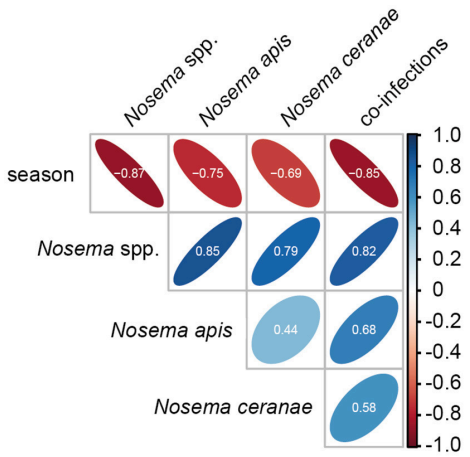


**FIGURE 3 |** Spearman correlation matrix values showing the pairwise correlation coefficients for the parameters season and the four infection categories (*Nosema* spp.-, *N. apis*-, *N. ceranae*-, and co-infections). Shape and orientation of the ellipses represent the data clouds of the respective correlation coefficients. Positive correlations are illustrated in blue and negative correlations in red. Coefficients (white digits) between |0.10| and |0.29| represent weak associations, coefficients between |0.30| and |0.49| represent medium associations and coefficients of |0.50| or above (beyond) represent strong associations.

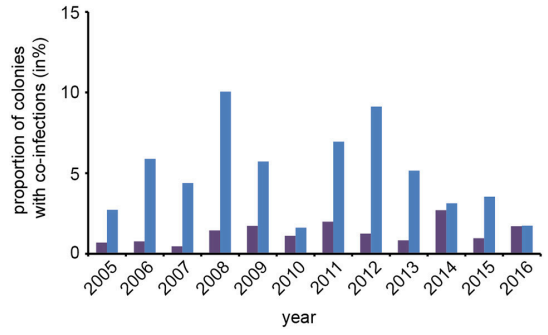


**FIGURE 4 |** Predicted and observed frequencies of prevalence of colonies being co-infected in spring with *N. apis* and *N. ceranae*. Predicted values for co-infections (violet bars) were calculated from the observed frequencies of *N. apis*- and *N. ceranae*-infected colonies and compared to the observed values for co-infection prevalence (blue bars) for each spring between 2005 and 2016.

*N. ceranae*-infections was significantly (GLM, Likelihood Ratio test of the model, $p = 0.0003$) increasing by an average of about 15 % per year (Odd Ratio: 1.15 [1.07, 1.25]) over the study period (**Figure 6B**) while at the same time the prevalence of *N. apis*-infections was significantly decreasing by an average of about 11% per year (Odd Ratio: 0.89 [0.84, 0.95]) (GLM, Likelihood Ratio test of the model, $p = 0.0003$) (**Figure 6C**). For co-infections, however, no significant (GLM, Likelihood Ratio

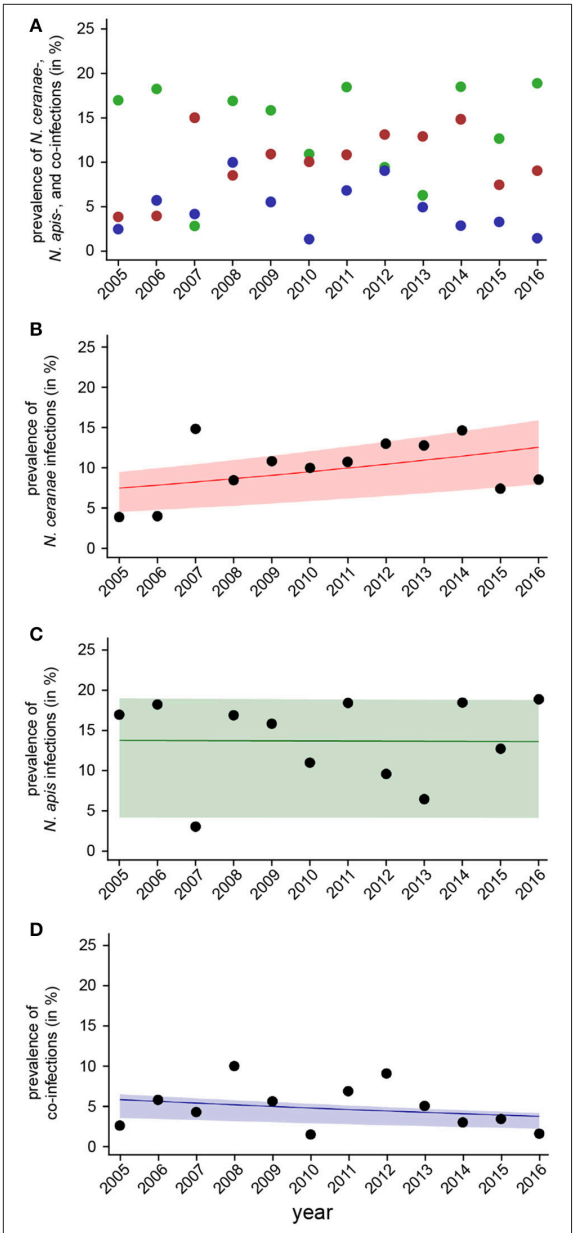


**FIGURE 5 |** Temporal patterns of prevalence of single infections with *N. ceranae* or *N. apis* and of co-infections detected in spring samples between 2005 and 2016. **(A)** Prevalence data for *N. ceranae*-, *N. apis*-, and co-infections in spring are plotted against year. **(B)** Data sets for prevalence of *N. ceranae*-, *N. apis*-, and co-infections in spring were fitted by Linear Mixed-Effects Models to visualize the relationship between the independent variables (year) and the dependent variables *N. ceranae*- **(B)**, *N. apis*- **(C)**, and co-infections **(D)**. Regression lines visualizing trends for *N. ceranae*- (**B**; solid red line), *N. apis*- (**C**; solid green line), and co-infection (**D**; solid blue line) prevalence are shown; ribbons represent the 2nd and 3rd quartile (25–75%) of the predicted data of the model.

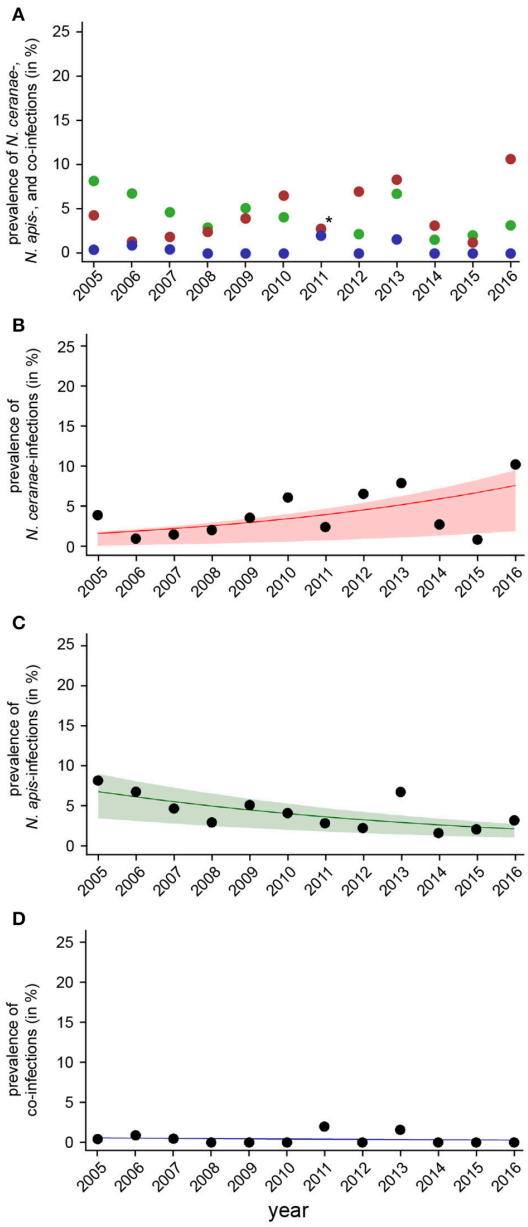


**FIGURE 6 |** Temporal patterns of prevalence of single infections with *N. ceranae* or *N. apis* and of co-infections detected in autumn samples between 2005 and 2016. **(A)** Prevalence data for *N. ceranae*-, *N. apis*-, and co-infections in autumn are plotted against year. In autumn 2011, highlighted by an asterisk, the proportions of *N. apis*- and *N. ceranae*-infections were identical, hence, only a red dot is visible. **(B)** Data sets for prevalence of *N. ceranae*-, *N. apis*-, and co-infections in autumn were fitted by Linear Mixed-Effects Models to visualize the relationship between the independent variables (year) and the dependent variables *N. ceranae*- **(B)**, *N. apis*- **(C)**, and co-infections **(D)**. Regression lines visualizing trends for *N. ceranae*- (**B**, solid red line), *N. apis*- (**C**; solid green line), and co-infection (**D**; solid blue line) prevalence are shown; ribbons represent the 2nd and 3rd quartile (25–75%) of the predicted data of the model.

**TABLE 2 |** Results of the binary logistic regression analysis of prevalence of *N. apis*-, *N. ceranae*- and co-infections over the 12 year study period (see also **Figures 5**, **6**).

| | Infection categories | Odd Ratios (CI)* | *p*-value |
|---|---|---|---|
| spring | *N. apis*-infections | 0.00 (0.96, 1.04) | 0.95 |
| | *N. ceranae*-infections | 1.05 (1.01, 1.10) | 0.02 |
| | co-infections | 0.96 (0.90, 1.02) | 0.17 |
| autumn | *N. apis*-infections | 0.89 (0.84, 0.95) | 0.0003 |
| | *N. ceranae*-infections | 1.15 (1.07, 1.25) | 0.0003 |
| | co-infections | 0.95 (0.81, 1.10) | 0.49 |

*, *95% confidence interval.*

test of the model, $p = 0.5$) change in prevalence could be demonstrated between 2005 and 2016 (Odd Ratio: 0.95 [0.81, 1.11]) (**Figure 6D**).

## *In vitro*-Infection of IPL-LD-65Y Cells

To explain the obvious success of *N. ceranae* over *N. apis* in the studied honey bee population in summer, we experimentally analyzed the proliferative capacity of both microsporidian species in infected cells at temperatures between 21° and 33°C. To this end, we used an established cell culture model for *N. apis* and *N. ceranae* based on experimentally infecting cultured IPL-LD-65Y-cells. This insect cell line derived from *Lymantria dispar* had been shown to support replication of both microsporidian species (Gisder et al., 2011).

Intracellular proliferation of *N. apis* and *N. ceranae* at three different temperatures (21°, 27°, and 33°C) was evaluated by determining the number of the developmental stages per cell produced during merogony (meronts) and sporogony (sporonts/spores) of *Nosema* spp. (**Figure 7A**). The number of both meronts and sporonts/spores increased for *N. apis* as well as for *N. ceranae* over the observation time period of 96 h at all three tested temperatures. However, the number of the different developmental stages varied between *N. apis* and *N. ceranae* infected cells depending on incubation time and incubation temperature. At 21°C, there was no significant difference in the proliferative capacity of *N. ceranae* and *N. apis* in infected cells for both meronts and sporonts/spores at all tested time points (24, 32, 48, 72, and 96 h post-infection) (all $p > 0.05$) (**Figure 7B**). However, at 27°C and even more so at 33°C, a higher proliferation rate and a faster proliferation of *N. ceranae* compared to *N. apis* could be observed. In infected cells which were incubated at 27°C (**Figure 7C**), the number of meronts was not significantly different between *N. apis* and *N. ceranae* after 32 and 72 h post-infection ($p > 0.05$) but was significantly different at time points 24, 48, and 96 h post-infection ($p > 0.05$). More

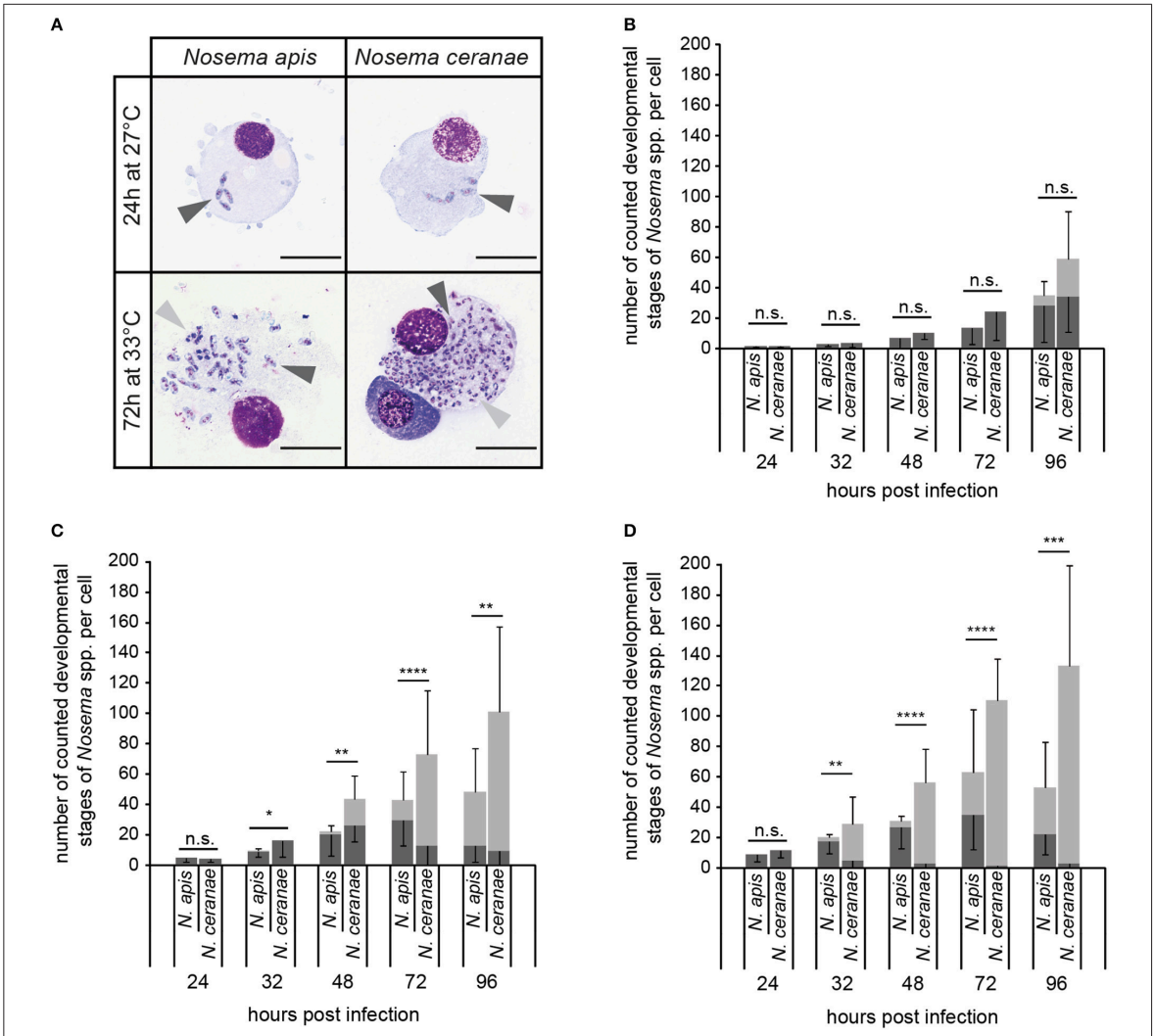


**FIGURE 7 |** *In vitro*-infection of IPL-LD-65Y cells with *N. apis*- and *N. ceranae*-spores. *Nosema* spp. proliferation was determined by counting the number of developmental stages of merogony (**A**; meronts, dark gray arrow heads) and sporogony (**A**; sporonts and intracellular spores, light gray arrow heads) in infected cells (scale bars = 25 μm) incubated at 21°C **(B)**, 27°C **(C)**, and 33°C **(D)**. Dark gray bars represent the number of meronts per cell (mean of 10 cells ± *SD*), light gray bars represent the number of sporonts/spores per cell (mean of 10 cells ± *SD*). Statistical analysis of the number of developmental stages was performed with student's *t*-tests for each time point and temperature. Statistical results given above the bars refer to the comparison of sporonts/spores produced by *N. apis* and *N. ceranae* (not significantly different: n.s., $p \geq 0.05$; significantly different: *, $0.05 < p < 0.01$; **, $0.01 < p < 0.001$; ***, $0.001 > p \geq 0.0001$; **** $p < 0.0001$).

importantly, the number of counted sporonts/spores at 32, 48, 72, and 96 h post-infection was significantly higher ($p < 0.05$) in *N. ceranae*- than in *N. apis*-infected cells (**Figure 7C**). When the host cells were incubated at 33°C, the number of *N. apis* meronts was significantly (all $p < 0.01$) higher than the number of *N. ceranae* meronts at 32, 48, 72, and 96 h post-infection (**Figure 7D**) and the numbers of sporonts/spores were significantly higher at 32, 48, 72, and 96 h post-infection (all $p < 0.01$) in *N. ceranae*-infected host cells than in cells infected with *N. apis*.

## DISCUSSION

### Prevalence of *N. ceranae* Infections Follows the Same Seasonality as *N. apis* Infections

*Nosema ceranae* is an emergent pathogen of the Western honey bee *A. mellifera*. Its first detection in colonies of *A. mellifera* dates back a decade (Higes et al., 2006; Huang et al., 2007), although, it obviously switched host from *A. cerana* to *A. mellifera* about 40

years ago (Teixeira et al., 2013) and is now endemic in the global *A. mellifera* population. Several differences between *N. ceranae* and its congener *N. apis* have been reported, including differences in virulence, in seasonality of infections, and in temperature dependence of spore germination and biotic potential. Most of these differences seem to work in favor for *N. ceranae* resulting in its continous spread in the bee population and a supersession of *N. apis* in many regions (Klee et al., 2007; Chen et al., 2008; Williams et al., 2008; Invernizzi et al., 2009; Chen and Huang, 2010; Stevanovic et al., 2011; Yoshiyama and Kimura, 2011; Copley et al., 2012). However, studies claiming lack of seasonality of *N. ceranae* or replacement of *N. apis* in a given honey bee population are rather short-termed studies rarely performed over more than 2 years and most often involving only a limited set of samples. Our epidemiology data based on observing a cohort 230 bee colonies sampled twice a year over 12 years now revealed a different picture at least for the study area.

*Nosema apis*-infections are known to follow a seasonal pattern with spring prevalence being higher than autumn prevalence. This seasonality can be explained by the pathobiology of *N. apis*: (i) Only the spores of *N. apis* are infective; (ii) older bees are more likely to be infected and carry more spores; and (iii) spores are most efficiently transmitted through the fecal-oral route (Bailey, 1967; Bailey and Ball, 1991). Therefore, *N. apis* transmission within the colony is favored by conditions with low or no brood rearing and forcing adult bees to stay inside the hive for longer periods and to have close in-hive contacts (Bailey, 1967; Bailey and Ball, 1991). These conditions are regularly fulfilled during the winter months in climatic zones with winter temperatures falling below 10°C not allowing bees to fly out (Winston, 1987). Instead, in these regions honey bee colonies hibernate by longlived adult winter bees forming a winter cluster around the queen bee and not leaving the hive for weeks or months until weather conditions allow cleansing and foraging flights and restarting brood rearing to replace the old winter bees (Winston, 1987). This explains why *N. apis*-infection levels increase over winter but normally decrease over summer when the rather shortlived summer bees are engaged in foraging, are able to defecate outside the hive, and when newly raised bees regularly replace older more heavily infected bees (Bailey, 1967; Bailey and Ball, 1991; Retschnig et al., 2017).

In contrast, *N. ceranae*-infections were described to lack this characteristic seasonality (Higes et al., 2006, 2010) suggesting fundamental differences in pathobiology and preferred routes of transmission which would be interesting to investigate. To analyse this suggested lack of seasonality, we collected bee samples in spring and autumn without gap over 12 years from a cohort of around 230 honey bee colonies and analyzed all samples for the presence of *Nosema* spp. spores and performed molecular species differentiation in all *Nosema* spp.-positive samples. Surprisingly, the data clearly disproved that *N. ceranae*-infections differ from *N. apis*-infections in regard to seasonality. Quite the contrary was true: All four infection categories, *Nosema* spp.-, *N. apis*-, *N. ceranae*-, and co-infections, followed the same seasonal pattern with spring prevalence of infection regularly being higher than autumn prevalence suggesting that *N. ceranae* and *N. apis* circulating in Northeast Germany are similar in regard to pathobiology and preferred transmission routes. Since reports on the lack of seasonality predominantly stem from South Europe (Higes et al., 2006, 2010), further experimental studies are necessary to analyse whether the differences in seasonality between the Northern and Southern parts of Europe are due to climatic factors or intraspecies differences in *N. ceranae*.

## No Evidence for a General Advantage of *N. ceranae* Over *N. apis* and for an Overall Replacement of *N. apis* by *N. Ceranae*

In many regions of the world, prevalence data collected for *N. apis* and *N. ceranae* indicated that *N. ceranae* has become the dominant species in the worldwide honey bee populations and it was suggested that *N. ceranae* has replaced or is about to replace its congener globally (Chen et al., 2012; Martin-Hernandez et al., 2012). However, in Europe, a South to North gradient was observed with *N. ceranae* being dominant in Southern European countries already 10 years ago while at that time *N. apis* was still dominant in the Northern part of Europe (Klee et al., 2007) which might reflect an already discussed climatic aspect in *N. ceranae* spread and assertiveness (Fenoy et al., 2009; Martin-Hernandez et al., 2009; Gisder et al., 2010; Chen et al., 2012; Natsopoulou et al., 2015).

Congruent with this South to North gradient (Klee et al., 2007), at the beginning of our epidemiology study we observed very low levels of prevalence for *N. ceranae*-infections in Northeast Germany compared to *N. apis*-infections. This starting condition, the size of the cohort, and the design and duration of the study provided a unique opportunity to follow the spread of the emerging pathogen *N. ceranae* and analyse the impact of this spread on its congener *N. apis*, well established in the observed honey bee population. Our epidemiology data show that starting from a very low level, the prevalence of *N. ceranae*-infections significantly increased continuously in the observed cohort of honey bee colonies during the last 12 years. This increase was true for both time points of sampling, in spring (showing the development over winter) and autumn (showing the development over summer) clearly indicating that *N. ceranae* became successfully established and expanded its presence in the honey bee population of Northeast Germany.

With regard to replacement of *N. apis* by *N. ceranae*, the obtained epidemiology data showed a complex picture. For assuming a replacement process at the population level, *N. apis* infection prevalence should have concomitantly decreased during the study period. However, a significant decrease in *N. apis*-infection prevalence was only observed for autumn indicating that during the bee season in summer *N. ceranae* successfully competed with *N. apis* at the population level over the course of the study. Surprisingly and in contrast to autumn, no significant change in *N. apis* infection prevalence was evident in spring despite a significant increase in *N. ceranae* prevalence. Therefore, no replacement of *N. apis* by *N. ceranae* in the honey bee population of Northeast Germany took place over winter during the last 12 years. Instead, the increase in *N. ceranae* prevalence in spring came on top of the unaltered *N. apis* infection prevalence suggesting that the two microsporidian

parasites did not compete with each other over winter at the colony and population level. In addition, the long term stability of *N. apis*-infection frequency in spring indicate, that whatever mechanisms are acting on *N. apis* during summer and causing its decrease in the population, they are compensated for and reversed during winter preventing a supersession of *N. apis* through *N. ceranae* in the observed honey bee population.

Replacement of *N. apis* by *N. ceranae* at the population level during summer but not during winter points to different mechanisms acting on or influencing the two microsporidian parasites in summer and over winter. Although the exact mechanism responsible for presence (summer) and absence (winter) of replacement at the population level still remain elusive, experimental data providing explanations at the individual bee level for the increase in *N. ceranae* infection prevalence over summer exists. In a recent study by Martin-Hernandez et al. (2009), infection experiments with caged bees were performed at different temperatures and the "biotic index" was calculated for both microsporidia as the total *N. apis* or *N. ceranae* spore count per day after infection. This "biotic index" was higher for *N. ceranae* than for *N. apis* at 25°C but no significant difference could be observed at 33°C (Martin-Hernandez et al., 2009). Although these results did not provide convincing proof for an advantage of *N. ceranae* over *N. apis* during summer, they pointed into an interesting direction. Therefore, we extended the approach and performed infection experiments in cell culture (Gisder et al., 2011), which allowed a detailed analysis of the time course of proliferation and of the proliferative potential of *N. ceranae* and *N. apis* at different temperatures. Our *in vitro* results revealed a significant advantage of *N. ceranae* over *N. apis* at 27° and 33°C, the normal range of daily maximum temperatures in summers in Northeast Germany. At both temperatures, *N. ceranae* completed its replicative cycle faster and replicated more efficiently than *N. apis*. These results were in accordance with a recent study, suggesting a generally higher proliferation rate for *N. ceranae* compared to *N. apis* in experimentally infected, caged bees incubated at 30°C for 20 days (Huang and Solter, 2013). Earlier and higher production of spores, which are transmitting the disease within and between colonies, may translate into higher infection prevalence at population level. These data explain an increase of *N. ceranae* infection levels, however, they still do not explain the observed replacement of *N. apis* by *N. ceranae* over summer.

For replacement of *N. apis* by *N. ceranae*, a simple increase in *N. ceranae* infection prevalence is not sufficient but a successful interspecies competition, with *N. ceranae* at least more often than *N. apis* winning the game, is necessary. Again, only experimental data at the individual bee level are available. Co-infection experiments with caged bees and simultaneous feeding of *N. apis* and *N. ceranae* spores did not provide evidence for intra-host competition between the two species (Forsgren and Fries, 2010; Milbrath et al., 2015). In contrast, sequential feeding of spores of the two species resulted in within-host competition: The first parasite significantly inhibited the growth of the second, regardless of species (Natsopoulou et al., 2015). This would have prevented the spread of *N. ceranae* because *N. apis* had been present in the bee population before *N. ceranae* arrived and would always have been first. However, this so-called "priority effect" proved to be asymmetric and *N. ceranae* exhibited a stronger inhibitory effect on *N. apis* than *N. apis* on *N. ceranae* (Natsopoulou et al., 2015). Mathematical modeling proposed that this priority effect will result in a successful replacement process at population level even when taking into account that the cold sensitivity of *N. ceranae* but not of *N. apis* spores (Fenoy et al., 2009; Gisder et al., 2010) provides a disadvantage for *N. ceranae* during cold winters (Natsopoulou et al., 2015).

However, for spring samples our epidemiology data clearly showed that although *N. ceranae*-infection prevalence increased over time, this increase did not result in a replacement of *N. apis*. Remarkably, *N. apis*-infection prevalence in spring remained rather stable over the 12 years study period although the autumn infection prevalence and, hence, the infection prevalence at the beginning of winter, has been declining during this period. Therefore, the two *Nosema* species rather not competed during winter and the mechanisms promoting the increase of *N. ceranae* in the studied honey bee population over winter did not influence the prevalence of *N. apis*.

Furthermore, we observed higher than expected co-infection rates in spring suggesting that there is no interspecies within-host competition at colony or population level during overwintering. The co-infection levels rather suggested that an infection with any one of the two microsporidia pre-existing in a colony favored an additional infection of the colony with the other microsporidium. This is in contrast to the above mentioned report (Natsopoulou et al., 2015) showing interspecies within-host competition with a priority effect favoring the spread *N. ceranae* over *N. apis*. However, this inter-species competition was shown at the individual bee level whereas our epidemiology data concern the colony and population levels. And indeed, at the colony and population level it is hardly conceivable how an *N. ceranae* infection of one bee or colony might inhibit a nestmate or a neighboring colony, respectively, to become infected by *N. apis* - and the other way round. Actually, the concept of interspecies within-host competition of an obligate intracellular parasite due to competition for the same limited cellular energy resources cannot easily be translated to the colony or population level where limitation or shortage of resources (in this case: new hosts) is not yet the problem. However, if the prevalence of *N. ceranae*-infections keeps increasing like it did over the last 12 years, within-colony and between-colony competition might become an issue once all colonies are infected with either one of the microsporidia. Therefore, a continuation of this study will further our understanding of the long term epidemiology and interspecies competition at population level of these two important honey bee pathogens.

## AUTHOR CONTRIBUTIONS

EG and SG conceived and designed the study and the experiments. SG, VS, and LH carried out the experiments and the microscopic and molecular diagnosis of *Nosema* spp. SG, VS, and DG performed the statistical analysis. EG supervised all work,

SG supervised the laboratory experiments, and DG supervised the statistical analysis. SG and EG wrote the paper. All authors revised the manuscript and approved the final version.

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## 2.2 Statistical significance and biological relevance: The case of *Nosema ceranae* and winter colony losses

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My contribution to this publication was mainly the data curation, data transformation, statistical analysis, and visualization (Figs. 1-5). For these tasks, I supervised a student assistant (Yuk-Chien Lui). During the last three years of the study period, I performed both the microscopic and molecular diagnostics of *Nosema* spp. and the determination of *V. destructor* infestation. Thereby, I was able to contribute and implement my own ideas. I was completely involved in discussing the results, the refining of the analysis and the preparation of the manuscript. I worked on the content revision of the manuscript and approved the submitted version.

**Statistical significance and biological relevance: The case of *Nosema ceranae* and winter colony losses.**

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Running title: *N. ceranae* affecting honey bee colonies

**Abstract**

Managed and wild insect pollinators play a key role in ensuring that mankind is adequately supplied with food. Among the pollinating insects, the managed Western honey bee providing about 90% of commercial pollination is of special importance. Hence, diseases as well as disease causing pathogens and parasites that threaten honey bees, have become the focus of many research studies. The ectoparasitic mite *Varroa destructor* together with deformed wing virus (DWV) vectored by the mite have been identified as the main contributors to colony losses, while the role of the microsporidium *Nosema ceranae* in colony losses is still controversially discussed. In an attempt to solve this controversy, we statistically analyzed a unique data set on honey bee colony health comprising data on mite infestation levels, *Nosema* spp. infections and winter losses continuously collected over 15 years. We used various statistical methods to investigate the relationship between colony mortality and the two pathogens, *V. destructor* and *N. ceranae*. Our multivariate statistical analysis confirmed that *V. destructor* is the major cause of colony winter losses. When using cumulative data sets, we also found a significant relationship between *N. ceranae* infections and colony losses. However, determining the effect size revealed that this statistical significance was of low biological relevance, because the deleterious effects of *N. ceranae* infection are normally masked by the more severe effects of *V. destructor* on colony health and therefore only detectable in the few colonies that are not infested with mites or are infested at low levels.

**Keywords:** honey bee colony losses, Varroa destructor, mite infestation, Nosema ceranae, Nosema epidemiology, effect size and biological relevance

48 **Introduction**

49 The basis of human nutrition includes agriculturally grown crops and fruits, many of which
50 are dependent on insect pollination for fruit set, seed production, and yield. Managed and
51 wild insect pollinators therefore play a key role in ensuring that mankind is adequately
52 supplied with food (Aizen et al., 2008; Aizen et al., 2009; Garibaldi et al., 2013). As a result,
53 the health and survival of pollinating insects have attracted increasing public and scientific
54 interest and consequently diseases as well as disease causing pathogens and parasites that
55 threaten pollinating insects have become the focus of many research studies. In terms of
56 pollinating insects, the main focus is on the Western honey bee *Apis mellifera*, which is
57 managed by beekeepers for honey production all over the world and provides 90% of the
58 commercial pollination worldwide (Aizen et al., 2008). In terms of pathogens, the focus is on
59 those that threaten the survival of the managed honey bee colonies. The ectoparasitic mite
60 *Varroa destructor* together with deformed wing virus (DWV) vectored by the mite have been
61 identified as the main contributors to colony losses (Dainat et al., 2012; Dainat and
62 Neumann, 2013; Genersch et al., 2010; van Dooremalen et al., 2012). The microsporidium
63 *Nosema ceranae* (*N. ceranae*) has also been implicated in regional colony losses (Botías et
64 al., 2013; Fries et al., 2006; Higes et al., 2008; Higes et al., 2009; Martin-Hernandez et al.,
65 2007). The threat posed by these pathogens is compounded by the fact that honey bee
66 colonies are usually infected by several pathogens simultaneously, with *V. destructor*
67 (together with DWV) and *Nosema* spp. being the most widespread and therefore often
68 occurring together.
69 The mite *V. destructor* is an ectoparasite of honey bees (*Apis mellifera*, *A. cerana*) that
70 infests honey bee colonies all over the world (for a recent review on *V. destructor* please see
71 (Traynor et al., 2020)). The life cycle of *V. destructor* in honey bee colonies is divided into
72 two phases, (i) the dispersal phase in which adult female mites parasitize adult bees and use
73 the bees as a means of transport and (ii) the reproductive phase that takes place in the
74 capped brood cell (Rosenkranz et al., 2010). For reproduction, a mature mated female mite
75 enters a brood cell shortly before cell capping and starts laying eggs and raising her offspring

soon the larva has reached the prepupal stage. For feeding, the mother mite punctures a hole in the cuticle of the developing bee. This hole is then the feeding site for the growing mite family and allows access to the pupa's fat body, which serves as nutritional resource (Ramsey et al., 2019). Bees developing from *V. destructor* parasitized pupae show accelerated behavioral maturation, resulting in a shortened phase as nurse bees (Zanni et al., 2018), contribute less to colony productivity, and have a reduced longevity (Rosenkranz et al., 2010). Heavily mite infested colonies are characterized by an increasing rate of emerging bees which are not viable and have crippled wings. Initially, these symptoms were thought to be caused solely by mite parasitization, but it soon became clear, that *V. destructor* is an efficient virus vector (Ball, 1983; Ball, 1989) and that the crippled wings syndrome was caused by a virus, which was then named deformed wing virus (DWV) (Bailey and Ball, 1991; Bowen-Walker et al., 1999). We now know that at least four major variants of DWV exist (de Miranda et al., 2022; Martin et al., 2012; Mordecai et al., 2016; Ongus et al., 2004) and that it is the variant DWV-B that causes most of the symptoms, is more virulent than the DWV-A, and uses *V. destructor* as biological vector (Gisder et al., 2009; Gisder and Genersch, 2021; Gisder et al., 2018; McMahon et al., 2016; Posada-Florez et al., 2019; Yue and Genersch, 2005). Although *V. destructor* itself is sufficient to cause considerable damage to the parasitized pupa and the infested colony, it is the mite-vectored viruses, particularly deformed wing virus (DWV), that exacerbate the damage and link mite infestation to colony losses especially during the winter season (Dainat and Neumann, 2013; Genersch et al., 2010; Martin, 2001; Martin et al., 2012; Martin et al., 1998; McMahon et al., 2016).

Microsporidia are fungal related, obligate intracellular parasites that infect many vertebrate and invertebrate host species (Keeling and Fast, 2002). Two microsporidian species infecting the adult Western honey bee *A. mellifera* are described: *Nosema apis* and *N. ceranae*. While *N. apis* is known as a honey bee-specific pathogen since more than 100 years (Zander, 1909), *N. ceranae* was originally described as pathogen of the Eastern honey bee *Apis cerana* (Fries et al., 1996), but obviously switched host several decades ago and by now is even more prevalent than *N. apis* in many *A. mellifera* populations (Botías et al., 2012;

Chauzat et al., 2007; Higes et al., 2010; Klee et al., 2007; Paxton et al., 2007). However, a recent study showed that there is still no general replacement of *N. apis* by *N. ceranae*, but replacement seems to be a rather regional phenomenon (Gisder et al., 2017), presumably influenced in its dynamics by climatic conditions since *N. ceranae* spores quickly lose their infectivity when exposed to low temperatures (Fenoy et al., 2009; Gisder et al., 2010; Martin-Hernandez et al., 2009). From a clinical point of view, there is not much difference between *N. apis* and *N. ceranae*: Both pathogens frequently cause asymptomatic infections of the midgut epithelium of adult bees, but infections can also lead to diarrhea (Horchler et al., 2019). However, the factors causing the switch from asymptomatic to symptomatic infections are poorly understood (Fries, 1993; Fries, 2010). Symptomatic outbreaks of *Nosema* spp.-infections are called nosemosis and can be diagnosed by the characteristic fecal spots visible at the hive entrance and inside the hive (Horchler et al., 2019). These fecal spots contain millions of infectious spores and drive the fecal-oral transmission of the disease within the colony, as adult bees cleaning the hive of the spots ingest the spores and become infected (Bailey, 1967; Bailey and Ball, 1991). Infection in the individual adult bee host is initiated by germination of the ingested spores in the midgut lumen; germination is followed by extrusion of the polar tube, mechanical piercing of a cell by this polar tube, and injection of the sporoplasm into the cell through the polar tube (Bigliardi and Sacchi, 2001; Franzen, 2005). The reproductive cycle within the infected host cell takes about 96 hours (Gisder et al., 2011), goes through several stages (merogony, sporogony) and ends when the newly generated spores are released into the gut lumen by the bursting of the cell. These newly generated spores in the gut lumen are defecated and can infect naïve adult bees when they try to clean the hive from spore contaminated fecal spots (Bailey, 1955; Bailey, 1967; Bailey and Ball, 1991).

In 2008, the first study was published suggesting that even asymptomatic *N. ceranae* infections result in the collapse of honey bee colonies (Higes et al., 2008). Since then, numerous studies have been published demonstrating an association between *N. ceranae* infections and colony losses, but there are also many studies that failed to confirm this

association (Botías et al., 2013; Fernández et al., 2012; Fries et al., 2006; Guimarães-Cestaro et al., 2020; Guzman-Novoa et al., 2011; Higes et al., 2008; Higes et al., 2009; Martin-Hernandez et al., 2007; Stevanovic et al., 2011).

Among the studies that did not observe a statistically significant association between *N. ceranae* infection and colony losses is our long-term longitudinal cohort study on honey bee health in Northeast Germany, which was initiated in 2005 and is still ongoing (Gisder et al., 2010; Gisder et al., 2017). In addition to data on colony losses during the winter season and prevalence of *Nosema* spp.-infection in spring and autumn, *V. destructor* infestation levels were also determined in each autumn over the entire study period. This unique data set enabled us to investigate the relationship between colony mortality and the two pathogens that are most commonly blamed for colony losses, *V. destructor* and *N. ceranae*. Our multivariate statistical analysis confirmed that *V. destructor* is the major cause of colony winter losses, although *N. ceranae* infections can also have deleterious effects. However, these effects are normally masked by the more severe effects of *V. destructor* on colony health and therefore only detectable in colonies that are not infested with mites or are infested at low levels. With our results we end a long-running controversy about whether or not *N. ceranae* is capable to kill entire honey bee colonies and should be considered a serious threat or even an emerging infectious disease (EID) for honey bees.

## Results

### Winter mortality

Over the last 15 years, we performed a longitudinal cohort study on honey bee colony health in the honey bee population in Northeast Germany (Genersch et al., 2010; Gisder et al., 2010; Gisder et al., 2017). We monitored between 230 and 280 colonies each year throughout the entire study period and continously collected data on winter mortality as well as on *Nosema* spp. infection status and *Varroa destructor* infestation levels in the monitored colonies. The full data set for this study comprises 3502 colonies and is a uniquely solid basis for analyzing both, the dynamics of winter colony losses and the relation between

*Nosema* spp.-infection and *V. destructor*-infestation in autumn with colony losses in the following winter.

Within the study period, the winter colony loss rate varied from 4.8 % as the lowest value in the winter 2008/2009 to 26.0 % as the highest value in the winter 2016/2017. Using a linear model, we show that colony winter mortality increased by about 0.5 % per year in the studied cohort (Fig. 1A), but that this increase between 2005/2006 and 2019/2020 was not statistically significant (p-value of the F-statistic = 0.223, adjusted $R^2$ = 0.043). The mean of the average winter losses was 16.31 % ± 6.56 % (mean ± SD) and therefore statistically significantly higher (p-value= 0.0023; one-sample t-test) than the empirical threshold for acceptable winter mortality of 10% (Jacques et al., 2017).

We recently demonstrated that *N. ceranae* infection prevalence in autumn showed a statistically significant increase between 2005 and 2015 (Gisder et al., 2017). We again analyzed the dynamics of *N. ceranae* infection prevalence in autumn over the entire study period of meanwhile 15 years using a linear model and demonstrate a steady increase in *N. ceranae* infection prevalence which was statistically significant (p-value of the F-statistic = 0.021, adjusted $R^2$ = 0.295) and increased by about 0.5 % per year (Fig. 1B).

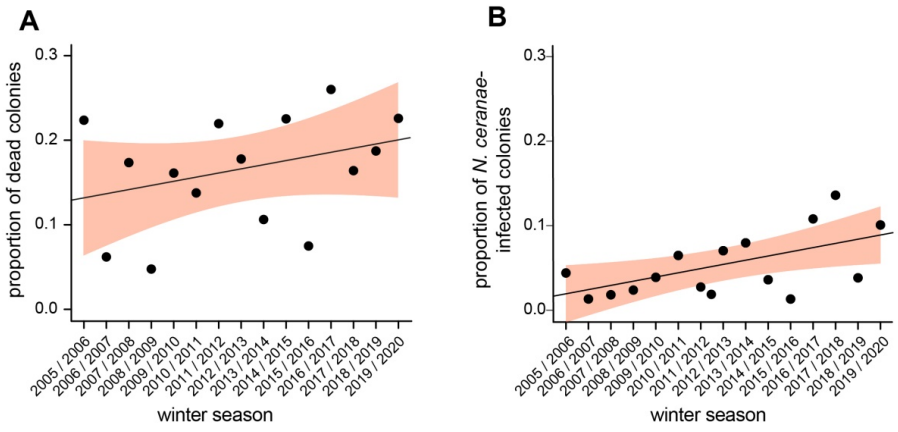


Figure 1: Dynamics of honey bee colony losses and *N. ceranae* infection prevalence between 2005 and 2020. Honey bee colony winter losses (A) and *N. ceranae* infection prevalence in autumn (B) were calculated over the study period from 2005 - 2020 with n=3502. Each data point represents the proportion of dead colonies in spring (A) or the prevalence of *N. ceranae*-infected colonies in autumn (B) per year. Linear regression models were calculated. Their regression lines are shown and their 95% CI (convidence interval) are highlighted in light red.

183

184 **Classification tree analysis**

185 We next aimed at identifying the factors responsible for the observed increased and
186 increasing winter losses in the monitored cohort of honey bee colonies. *V. destructor* has
187 been identified as the main pathogen driver of winter mortality in many studies, but *N.*
188 *ceranae* has also been implicated in colony losses. However, most of these studies are
189 based on univariate analyses that observe the effect of single explanatory variables on
190 colony mortality. Hence, data allowing to estimate the relative influence of *V. destructor* and
191 *N. ceranae* on colony mortality when both are present in a colony, are lacking. To close this
192 gap of knowledge, we used our data set comprising data on colony winter losses, *V.*
193 *destructor* infestation levels and *Nosema* spp.-infection status and performed a classification
194 tree analysis (Fig. 2), a tool of recursive partitioning for multivariate data exploration. This
195 multivariate analysis aimed at identifying if *V. destructor* infestation levels or *Nosema* spp.
196 infection status in autumn determined the fate of the colony over winter, hence, whether a
197 colony was prone to collapse over winter or had a realistic chance to survive.
198 A total of 3492 data sets was available for analysis. In the classification tree (Fig. 2), two
199 decision points describing the fate of the colony (survival or collapse) were identified. Both
200 were based on the mite infestation level indicating that the *Nosema* spp.-infection status of
201 the colonies were not identified by this analysis as decisive factor for colony collapse over
202 winter. The first decision point divided the analyzed colonies into two groups, one comprising
203 82% (n = 2861) of the colonies with a mite infestation rate in October of less than eight mites
204 per 100 bees and a mortality rate of 9.8 %. The remaining 18 % of the colonies (n = 631) had
205 an infestation level of eight or more mites per 100 bees and a mortality rate of 45.8 %. This
206 group was further subdivided into two groups, again based on the mite infestation level. 12 %
207 (n = 430) had an infestation level of eight or more mites but below 21 mites per 100 bees and
208 a mortality rate of 37.2 %, whereas the other branch comprised the remaining 6 % (n = 201)
209 of the colonies which were characterized by an infestation level of 21 or more mites per 100
210 bees and a mortality rate of 64.2 %. This classification tree showed convincingly that over the

study period of 15 years the strongest link was between colony winter losses and *V. destructor* infestation level, but not with *N. ceranae* or *N. apis* infection.

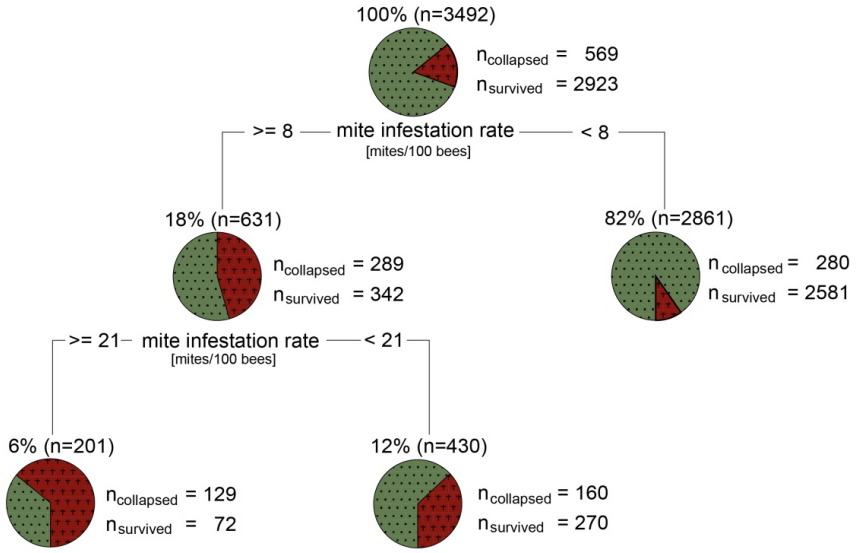


Figure 2: Major factors contributing to honey bee colony mortality visualized by a classification tree analysis using the R packages rpart (Therneau and Atkinson, 2019) and rattle (Williams, 2011) with standard settings.

### *N. ceranae* infection and winter mortality

The results from the classification tree analysis were in accordance with previous studies that did also not reveal any relation between colony winter losses and *Nosema* spp. infection (Genersch et al., 2010; Gisder et al., 2010; Gisder et al., 2017; Guzman-Novoa et al., 2011; Invernizzi et al., 2009; Williams et al., 2010), but contradicted other studies repeatedly showing that *N. ceranae* infections cause colony losses (Cepero et al., 2014; Higes et al., 2007; Higes et al., 2008; Higes et al., 2009; Martin-Hernandez et al., 2007). However, *Nosema* spp.-infections usually show a rather low prevalence in autumn (Bailey and Ball, 1991; Gisder et al., 2017), and hence our data set comprises only few colonies infected with *Nosema* spp. and even less infected with *N. ceranae* for each autumn. We, therefore, speculated that the number of *Nosema* spp. infected colonies per year might be too low to

see a relation between winter mortality and *Nosema* spp. infection and decided to generate higher numbers for *Nosema* infected colonies by summing the numbers for the individual years starting with autumn/winter 2005/2006 and ending with the sum of autumn/winter 2005/2006 up to autumn/winter 2019/2020 (Table 1). Forming these cumulative data subsets and examining time periods instead of annual values, resulted in larger group sizes (n's) and overall larger numbers of *Nosema* spp. infections, which made statistical analyses more robust (Table 1). We used a Chi-squared test to calculate the statistical relationship between infection status and winter mortality and indeed, the analysis started to become significant (p-value < 0.05) when more than 11 years (2005/2006 up to 2016/2017 resulting in more than 221 *Nosema* spp.-infected colonies among the analyzed 3492 colonies) were considered (Table 1, Fig. 3). This was the first time that we were able to show a statistically significant impact of *Nosema* infection on colony losses.

Table 1: Cumulative data subsets for determining the effects of *Nosema* spp.-infection in the autumn on honeybee colony losses in the following winter.

| Cumulative overwintering period | Total number of colonies | Surviving colonies | | Collapsed colonies | | p[a] |
|---|---|---|---|---|---|---|
| | | *Nosema* positive | *Nosema* negative | *Nosema* positive | *Nosema* negative | |
| 2005 | 237 | 21 | 163 | 9 | 44 | 0.28 |
| 2005 - 2006 | 463 | 40 | 356 | 10 | 57 | 0.24 |
| 2005 - 2007 | 682 | 51 | 526 | 14 | 91 | 0.15 |
| 2005 - 2008 | 892 | 62 | 715 | 14 | 101 | 0.13 |
| 2005 - 2009 | 1072 | 75 | 853 | 17 | 127 | 0.14 |
| 2005 - 2010 | 1319 | 96 | 1045 | 22 | 156 | 0.09 |
| 2005 - 2011 | 1574 | 111 | 1229 | 26 | 208 | 0.16 |
| 2005 - 2012 | 1844 | 133 | 1429 | 29 | 253 | 0.33 |
| 2005 - 2013 | 2070 | 166 | 1598 | 35 | 271 | 0.27 |
| 2005 - 2014 | 2292 | 175 | 1761 | 38 | 318 | 0.33 |
| 2005 - 2015 | 2519 | 182 | 1964 | 39 | 334 | 0.21 |
| 2005 - 2016 | 2769 | 203 | 2128 | 53 | 385 | 0.02 |
| 2005 - 2017 | 3019 | 234 | 2306 | 66 | 413 | < 0.01 |
| 2005 - 2018 | 3254 | 240 | 2491 | 73 | 450 | < 0.001 |
| 2005 - 2019 | 3502 | 267 | 2656 | 74 | 505 | <0.01 |

[a] Determined by the $\chi^2$ test

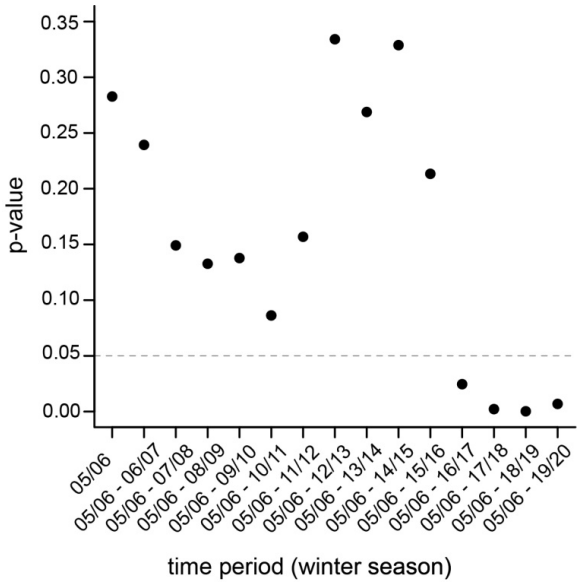


Figure 3: Calculated p-values for the relation between colony losses and Nosema-infection status using cumulated data for the numbers of infected clonies. Each data point represents the cumulative number of infected colonies summed over the time window indicated on the x-axis, while the y-axis gives the respective p-value of the Chi-squared test.

To further support and analyze this impact, we performed a Chi-squared test giving Pearson residuals using the data cumulated over the entire study period (2005/2006 to 2019/2020) for *Nosema* spp. infection (Fig. 4A) and for *N. apis*, *N. ceranae* and mixed infections separately (Fig. 4B). The black lines in the associations plots (Fig. 4) represent the expected values for the categories “alive” or “dead” in relation to the infection categories negative or positive (Fig. 4A) or negative and positive for *N. apis*-, *N. ceranae*- or co-infections (Fig. 4B). Overrepresented categories are represented by rectangles above the base line, while the rectangles for underrepresented categories are below the base line. Categories with Pearson residuals above 2.0 are shown in blue and appear only for the combinations “dead” and “positive for *Nosema* spp.-infection” (Fig. 4A) or “dead” and “positive for *N. ceranae* infection” (Fig. 4B). These results confirmed that the relation between Nosema infection and winter colony losses was statistically significant (p-value = 0.007). However, this was only true for *N. ceranae*-infections (p-value = 0.002); *N. apis*- or co-infections did not significantly contribute to winter losses.

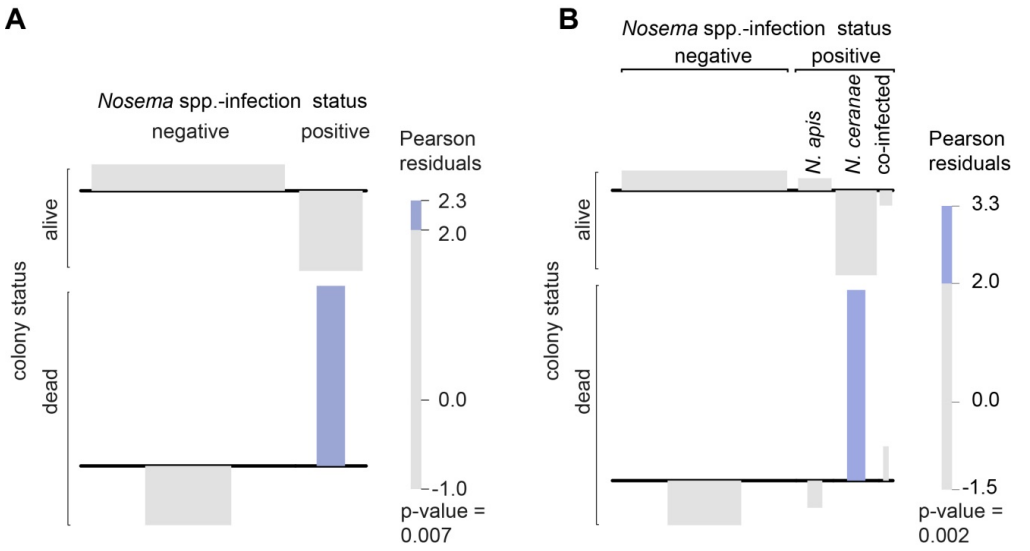


Figure 4: Contingency table analysis using association plots based on Chi-squared testing of the association between *Nosema* spp.-infection and colony mortality (A) and *N. apis*-, *N. ceranae* and co-infections and colony mortality (B). Overrepresented entries (dead/positive in A or dead/*N. ceranae* in B) are shown in blue.

**Statistical significance vs. biological relevance**

P-values are a measure of statistical significance, but are insufficient to show biological relevance. To analyze the biological relevance of our results, we therefore used the effect size measure Cohen's ω, which is applicable for two times two and larger contingency tables and is considered a measure of relevance with values above 0.1, 0.3, and 0.5 indicating a small, medium, and large effect size, respectively (Cohen, 1988). Calculating Cohen's ω for our data set and the relation between colony losses and *Nosema* spp.- or *N. ceranae*-infection revealed an effect size below 0.1, hence, a less than small effect (Fig. 5A). These results indicated that although we showed a statistically significant relationship between colony losses and *N. ceranae*-infection, these relationships are of minor or no biological relevance supporting the results of the classification tree analysis (Fig. 2) identifying *V. destructor* as main factor in colony losses.

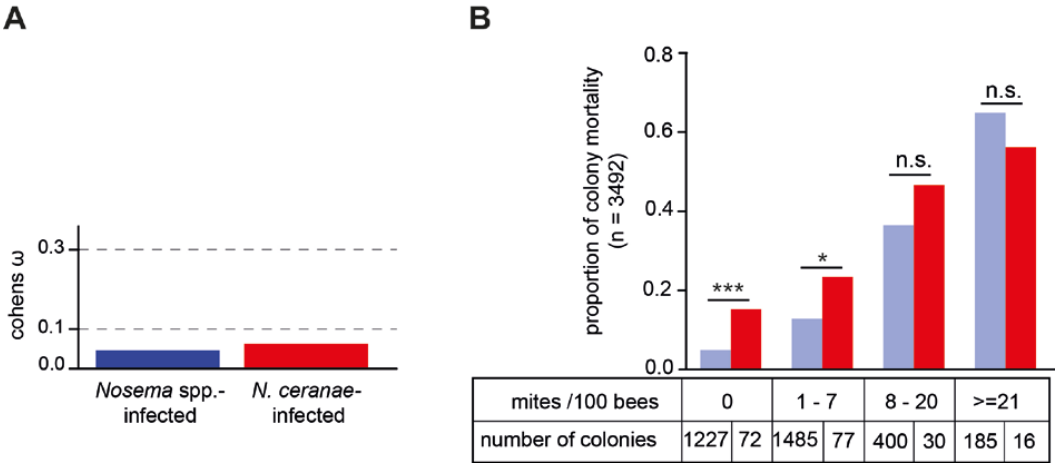


Figure 5: Biological relevance of *N. ceranae* infections. (A) Bar plot showing the effect size Cohen's ω for *Nosema* spp.-infected (dark blue) and *N. ceranae*-infected (red) colonies on colony mortality. Dashed lines indicate the conventional definition of Cohen's ω with a value for ω between 0.1 and 0.3 as small, between 0.3 and 0.5 as medium and above 0.5 as large effect (Cohen, 1988). (B) Bar plot showing the relation between colony mortality, mite infestation level categories and *N. ceranae*-infection (red bars) or *Nosema* spp.-infection excl. *N. ceranae*-infection (light blue bars). Significance levels are indicated by asterisks (n.s., $p \geq 0.05$; significantly different: *, $0.05 < p < 0.01$; **, $0.01 < p < 0.001$; ***, $0.001 > p$).

We therefore reanalyzed the data, but this time we considered the *V. destructor* infestation rate of the colonies by analyzing the mortality rate of colonies infected and not infected with *N. ceranae* within the mite infestation categories as defined by the classification tree (Fig. 2). This analysis revealed that *N. ceranae* infection only contributed siginficantly to colony mortality when the colonies did not harbor detectable *V. destructor* mites or very few mites (1-7 per 100 bees) in October. Only in these 149 out of 3474 colonies, *N. ceranae* was significantly correlated with winter mortality (Fig. 5B).

## Discussion

Honey bee colony losses and the quest for abiotic and biotic factors causing them are hot topics in the field of bee research since two decades. While it is widely accepted and unequivocally substantiated by many studies, that the ectoparasitic mite *V. destructor* and

316 the viruses vectored by the mite play a key role in winter colony losses, the role of *N.*
317 *ceranae* infections is less clear. There are studies clearly showing detrimental effects of *N.*
318 *ceranae* infection on honey bee colonies (Botías et al., 2013; Fries et al., 2006; Higes et al.,
319 2008; Higes et al., 2009; Martin-Hernandez et al., 2007). But there are also numerous
320 monitoring studies that fail to observe such an effect (Fernández et al., 2012; Gisder et al.,
321 2010; Gisder et al., 2017; Guimarães-Cestaro et al., 2020; Stevanovic et al., 2011). One
322 possible reason for this could be that in monitoring studies the damage caused by the almost
323 ubiquitous infestation of colonies with *V. destructor* masks the effects caused by pathogens
324 with rather low prevalance such as *N. ceranae*. This masking effect is difficult to see through
325 because most observational studies on colony losses include too few colonies and are
326 conducted over too short a time period to observe statistically significant associations for low-
327 prevalence pathogens. Such monitoring studies usually collect data for multiple pathogens
328 enabling to look for the interaction between co-existing pathogens. For example, *N. ceranae*-
329 infections in spring have been shown to correlate statistically significantly with an increased
330 prevalence of *Ascosphaera apis* infections and higher levels of *V. destructor* infestation in
331 summer (Hedtke et al., 2011). However, there are only few data on the relative impact of
332 individual pathogens on colony mortality, although it is widely accepted that colony collapse
333 is a multifactorial process, often likely involving multiple pathogens.
334 Our data on pathogen load and winter colony mortality, collected continuously over 15 years
335 from a relatively stable cohort of about 25 apiaries contributing ten colonies each is a unique
336 resource to study the role of *N. ceranae* on colony mortality, especially the relative impact of
337 *N. ceranae* infections on overwintering success of honey bee colonies, most of which were
338 concurrently infested by *V. destructor*. With more than 3000 data sets collected over 15
339 years, we were able to confirm that big data sets and long study durations are key for robust
340 analyses: Only by summing the data for more than 11 years did a statistically significant
341 association between *N. ceranae*-, but not *N. apis*-infection in the autumn and colony losses
342 the following winter become evident. No such relation was observed when the data were
343 analyzed year by year (Gisder et al., 2010; Gisder et al., 2017) because the prevalence of

*Nosema* spp.-infections in autumn and the mortality rate among these colonies are usually too low for statistical significance.

A statistically significant association between N. ceranae infection and colony losses was in accordance with studies that had suggested an increased virulence of *N. ceranae* compared to *N. apis* and the abilty of *N. ceranae* to cause the collapse of entire colonies (Botías et al., 2013; Fries et al., 2006; Higes et al., 2008; Higes et al., 2009; Martin-Hernandez et al., 2007). However, our multivariate data exploration via classification tree analysis had identified *V. destructor* infestation as the main variable explaining colony losses in our cohort. A result that is also in accordance with many other studies clearly linking mite infestation to winter colony mortality (Dainat and Neumann, 2013; Genersch et al., 2010; Martin, 2001; Martin et al., 2012; Martin et al., 1998; McMahon et al., 2016). Only with a mite infestation rate in October of less than eight mites per 100 bees, an acceptable winter mortality rate below 10 % (Jacques et al., 2017) can be reached. In the monitored cohort this was the case for 82 % of the colonies over the entire duration of the study, leaving 18 % of the cohort contributing to inacceptably elevated colony losses. Since classification tree analysis is the method of choice for determining biologically relevant factors and the classification tree identified mite infestation as the only relevant factor, it was not surprising that determination of Cohen's ω confirmed that the biological relevance of *N. ceranae* infection for colony losses is low despite the statistical significance of this association. This clearly shows that for biological questions the focus should rather not soley lay on statistical significance but more on the effect size and biological relevance.

We showed that *N. ceranae* infection contributed to colony losses only in those colonies that were not or low infested by *V. destructor* in autumn. As long as *V. destructor* infestation is the dominating health problem in honey bee colonies and *N. ceranae* prevalence is low, *N. ceranae* can be classified as pathogen causing little concern, because its role in colony losses is marginal. However, the situation might change when the prevalence of *N. ceranae* reaches a critical point. Since the increase in *N. ceranae* prevalence is continuing ( (Gisder et al., 2017) and this study), it is only a question of time when this point will be reached.

372 Monitoring not only mite infestation levels in colonies but also *N. ceranae* infection
373 prevalence in honey bee populations is therefore advisable. Hence, we will continue our
374 study and continuously calculate the effect size of *N. ceranae* infection on colony losses to
375 determine the critical prevalence of *N. ceranae* in a honey bee population.
376 Remarkable is that in an early report on colony collapse due to *N. ceranae* (Higes et al.,
377 2009) it was explicitly pointed out that mites were absent in all samples indicating a very low
378 number or even the total absence of *V. destructor* in these collapsed colonies due to efficient
379 mite control. The absence of *V. destructor* and concomitant presence of *N. ceranae* was the
380 most convincing argument for *N. ceranae* being the cause of colony collapse in the reported
381 case. Moreover, for experimentally demonstrating *N. ceranae*-induced colony collapse, the
382 colonies needed to be tightly controlled for *V. destructor* infestation (Higes et al., 2008).
383 These studies corroborate our results which indicate that *N. ceranae*-induced colony collapse
384 only becomes evident in (nearly) mite-free colonies. Hence, the more efficient the mite
385 control is, the more likely it is that *N. ceranae* induced damages become detectable. But
386 again, as long as *N. ceranae* prevalence is low this does not pose a serious threat because
387 *N. ceranae* is still not a highly virulent pathogen.
388
389 **Material and Methods**
390 **Bee Samples, Field Survey**
391 The data set of this study comprises samples which were collected from autumn 2005 to
392 spring 2020 in the course of a 15 year longitudinal cohort-study on *Nosema* spp.
393 epidemiology and honey bee health (Genersch et al., 2010; Gisder et al., 2010; Gisder et al.,
394 2017). Honey bee samples were collected in autumn and colonies were checked for their
395 survival in spring of the respective overwintering period (weeks 36 to week 14 of the
396 following year) from about 23 apiaries which were located in Northeast-Germany (Fig. 6).
397 Briefly, apiaries participated with ten so called “monitoring colonies” each. Apiaries or
398 monitoring colonies that dropped out during the study period were substituted by adequate
399 replacement. Hence, more than half of the apiaries (14 of ~23) participated for more than 9

years and 5 of them even for the entire duration of the study, *i.e.* 15 years. From at least 19 apiaries, samples were provided over a time period of consecutive 5-11 years (Fig. 6). This resulted in an annual mean of 23.4 ± 2.26 (mean ± SD) apiaries with 9.77 ± 1.25 (mean ± SD) colonies each, giving an overall count of n = 3502 sampled monitoring colonies which provide the basis of our analyses.

Sampling of bees was performed essentially as already described (Gisder et al., 2010; Gisder et al., 2017). Briefly, between calendar week 36 and 38 (late September/ beginning of October), about 300 in-hive honey bees were sampled from a super above the queen excluder (Fries et al., 2013) from each monitoring colony. Bee samples were stored at -20 °C until further analysis.

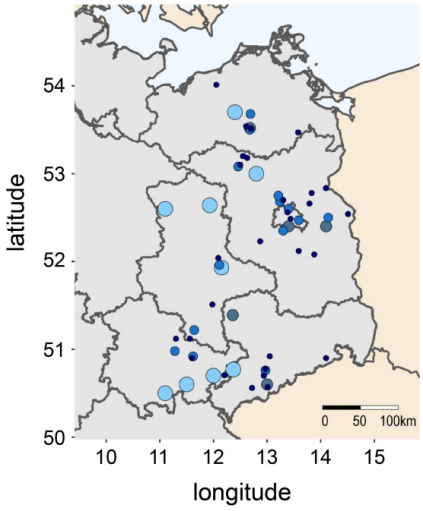


Figure 6: Map section of Northeast Germany showing the location of the apiaries which participated in the study. The size and color of the circles represent the number of years for which data are available for each apiary (light blue, 12 - 15 years; grey-blue, 9 - 11 years; royal blue, 5 - 8 years; dark blue, 1 - 4 years)

**Determination of mite infestation levels**

For determining the mite infestation level, *V. destructor* mites were washed from about 150 sampled bees following a standard protocol using a detergent solution (Dietemann et al., 2013). Briefly, the frozen bees were covered with soap and water in a jar. Subsequently, the

422 jar was shaken for 20 seconds and emptied into two stacked sieves with a white nylon cloth
423 between them. All mites were rinsed with plenty of water under high pressure through the
424 upper sieve which has larger aperture (3-4 mm) not allowing bees to pass. All mites were
425 collected on the cloth in the second sieve, which has smaller aperture (<0.5 mm) that no mite
426 fits through. To gain the mite infestation level in %, the number of counted mites was divided
427 by the number of sampled and washed bees multiplied by 100. Unfortunately, mite
428 infestation rate could not be determined in one apiary (ten colonies) in the first year (2005 /
429 2006) because of an insufficient number of honey bees available. Therefore, the dataset for
430 mite infestation level had to be reduced from 3502 to 3492.
431
432 **Diagnosis of *Nosema* spp. infection and molecular species differentiation**
433 Diagnosis of *Nosema* spp. infections was performed as already described (Genersch et al.,
434 2010; Gisder et al., 2010; Gisder et al., 2017) and in accordance with the "Manual of
435 Standards for Diagnostics and Vaccines" published by the Office International des Epizooties
436 (OIE), the World Organization for Animal Health (Anonymous, 2021). In short, per colony 20
437 pooled bee abdomens were homogenized and microscopically examined for the presence of
438 spores. Infection levels were determined by counting the number of *Nosema* spp. spores per
439 view field (three technical replicates each) in a hemocytometer (Neubauer-improved, VWR,
440 Darmstadt, Germany) using an inverse microscope (VWR, Darmstadt, Germany) with 100 ×
441 magnification. For classification of the infection levels, standard categories were used
442 (Anonymous, 2021): 0 (no spores), 1 (1-10 spores), 2 (11-100 spores), and 3 (more than 100
443 spores).
444 *Nosema* spp.-positive samples were subjected to further molecular species differentiation
445 either via PCR-amplification of a conserved region of the16S rRNA gene followed by RFLP
446 (restriction fragment length polymorphism) analysis of this amplicon (Gisder et al., 2010; Klee
447 et al., 2007) or via a species-specific duplex PCR-protocol taking advantage of species-
448 specific sequence differences in the highly conserved gene coding for the DNA-dependent
449 RNA polymerase II largest subunit (Gisder and Genersch, 2013). Molecular differentiation

enabled the distinction between single infections with either *N. apis* or *N. ceranae*, or co-infections where both infections are present at the same time (Table 2).

Table 2: Data on *Nosema* spp. epidemiology (prevalence of *Nosema* spp.-, *N. apis*-, *N. ceranae*- and co-infections) and winter losses collected between autumn 2005 and spring 2020

| winter season | total no. of colonies analyzed in autumn | no. of colonies between week 36 and 14 | | | | | | colony losses [%] |
|---|---|---|---|---|---|---|---|---|
| | | colonies alive in spring (survivors) | | | colonies dead in spring (winter loss) | | | |
| | | total | *Nosema* positive[a] | *Nosema* negative | total | *Nosema* positive | *Nosema* negative | |
| 2005 / 2006 | 237 | 184 | 21 (16, 4, 1) | 163 | 53 | 9 (3, 6, 0) | 44 | 22.4 |
| 2006 / 2007 | 226 | 212 | 19 (14, 3, 2) | 193 | 14 | 1 (1, 0, 0) | 13 | 6.2 |
| 2007 / 2008 | 219 | 181 | 11 (7, 3, 1) | 170 | 38 | 4 (3, 1, 0) | 34 | 17.4 |
| 2008 / 2009 | 210 | 200 | 11 (6, 5, 0) | 189 | 10 | 0 (0, 0, 0) | 10 | 4.8 |
| 2009 / 2010 | 180 | 151 | 13 (7, 6, 0) | 138 | 29 | 3 (2, 1, 0) | 26 | 16.1 |
| 2010 / 2011 | 247 | 213 | 21 (9,12, 0) | 192 | 34 | 5 (1, 4, 0) | 29 | 13.8 |
| 2011 / 2012 | 255 | 199 | 15 (6, 5, 4) | 184 | 56 | 4 (1, 2, 1) | 52 | 22.0 |
| 2012 / 2013 | 270 | 222 | 22 (4, 18, 0) | 200 | 48 | 3 (2, 1, 0) | 45 | 17.8 |
| 2013 / 2014 | 226 | 202 | 33 (16, 13, 4) | 169 | 24 | 6 (1, 5, 0) | 18 | 10.6 |
| 2014 / 2015 | 222 | 172 | 9 (4, 5, 0) | 163 | 50 | 3 (0, 3, 0) | 47 | 22.5 |
| 2015 / 2016 | 227 | 210 | 7 (5, 2, 0) | 203 | 17 | 1 (0, 1, 0) | 16 | 7.5 |
| 2016 / 2017 | 250 | 185 | 21 (6, 15, 0) | 164 | 65 | 14 (2, 12, 0) | 51 | 26.0 |
| 2017 / 2018 | 250 | 209 | 31 (6, 24, 1) | 178 | 41 | 13 (1, 10, 2) | 28 | 16.4 |
| 2018 / 2019 | 235 | 191 | 6 (1, 5, 0) | 185 | 44 | 7 (2, 4, 1) | 37 | 18.7 |
| 2019 / 2020 | 248 | 192 | 27 (2, 24, 1) | 165 | 56 | 1 (0, 1, 0) | 55 | 22.6 |

[a] The numbers of colonies positive for *N. apis*, for *N. ceranae*, and for both (mixed infections) are given in parentheses.

**Statistical Analysis**

Data was curated, transformed and presented in spreadsheets for the analysis with the statistic software R (R Development Core Team, 2021) using the package *openxlsx* (Schauberger et al., 2021). Data for the map section of Northeast Germany (Fig. 6) were obtained from the website http://www.gadm.org (Hijmans et al., 2016). The map was created with R by the use of the following packages: *rnaturalearth* (South, 2017), *raster* (Hijmans, 2022), *ggplot2* (Wickham, 2016), *sf* (Pebesma, 2018), *sp* (Bivand et al., 2013; Pebesma and Bivand, 2005), *rgeos* (Bivand and Rundel, 2021), and *reshape* (Wickham, 2007).

For the winter losses we performed a linear regression model (using R base package *stats*) and calculated its regression line, its slope, the adjusted $R^2$ and the F-statistic. To describe and visualize which variable(s) has (have) the largest share in honey bee colony mortality, we took advantage of classification tree analysis (decision trees), a tool of recursive partitioning for multivariate data exploration. We constructed a tree with default settings using *rpart* (Therneau and Atkinson, 2019) and *rattle* (Williams, 2011).

To understand the role of *N. ceranae* infection in more detail, we looked at the *Nosema* spp. infection frequencies using contingency tables and performed chi-squared calculation. The results were plotted in two-way association plots created by *vcd* (Meyer et al., 2006; Meyer et al., 2007; Meyer et al., 2020). The obtained results of the significance statistic were further assessed by strength statistic using the effect size index Cohen's ω (Cohen, 1988), which is an index for the biological effect size between two categorical variables. Cohen's ω is calculated as follows:

$$\omega = \sqrt[2]{\sum_{i=1}^{m} \frac{(p_{1i} - p_{0i})^2}{p_{0i}}}$$

$p_{1i}$ = the proportion in cell i posited by the alternate hypothesis and reflects the effect for that cell; $p_{0i}$ = the proportion in cell i posited by the null hypothesis; m = number of cells [ (Cohen, 1988) p. 216 formula 7.2.1]. For the interpretation of Cohen's ω in terms of effect size, there is a framework of conventional definition saying that $0.1 \leq \omega < 0.3$ is a small, $0.3 \leq \omega < 0.5$ is a medium and $\omega \geq 0.5$ is a large effect size [ (Cohen, 1988) chapter 7.3 p.227].

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

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My contribution to this publication was mainly the implementation of the exposure bioassay design (Fig. 1). I contributed to the execution of cage experiments, molecular diagnostics, preparation and analysis of microscopic samples, and FISH analysis (Fig. 2, Fig. 4). I took part in analysis and discussion of the results and I worked on the content revision of the manuscript and approved the published version.

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# 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, $P$

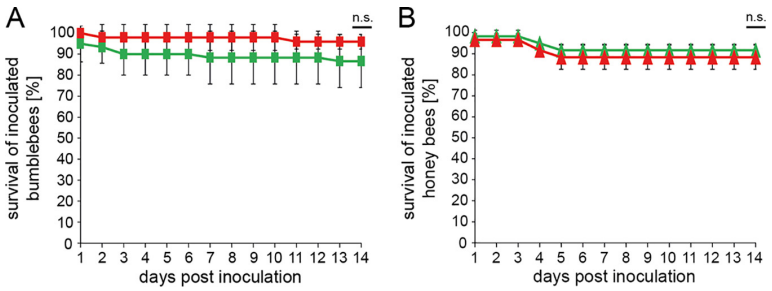


**FIG 1** Survival of *B. terrestris* and *A. mellifera* inoculated with *N. ceranae* spores. The experiments were perfomed in triplicate for each group. Newly emerged bumblebees ($n = 16$, 16, and 17, red rectangles) (A) and honey bees ($n = 20$ each replicate, red triangles) (B) were individually inoculated with 5 $\mu$l 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 $\mu$l 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 |

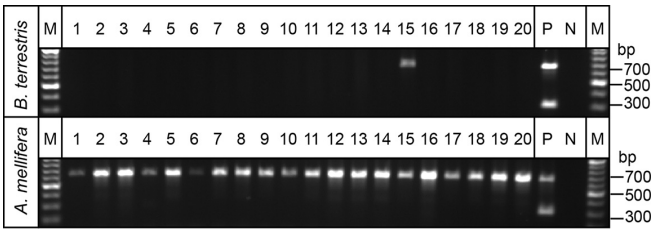


**FIG 2** Molecular detection of *N. ceranae* in orally inoculated bumblebees and honey bees using PCR. Newly hatched *B. terrestris* and *A. mellifera* workers were orally inoculated with 50,000 *N. ceranae* spores each, and molecular detection of *N. ceranae* via PCR in inoculated animals was performed 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% ± 0.21% (mean ± 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% ± 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).

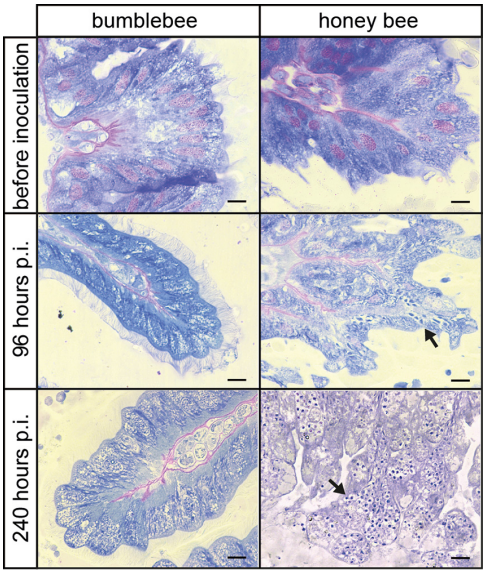


**FIG 3** Histological analysis of midgut sections of bumblebees and honey bees inoculated with *N. ceranae* spores. Midgut tissue sections of *B. terrestris* and *A. mellifera* inoculated with 50,000 spores per bee were Giemsa stained and microscopically analyzed for the intracellular presence of *N. 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% ± 16.1% [mean ± SD]) or 50,000 spores each (66.7% ± 28.9% [mean ± SD]) or 500,000 spores each (75.0% ± 5.0% [mean ± SD]) or were subjected to mock inoculation (83.6% ± 3.8% [mean ± 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.

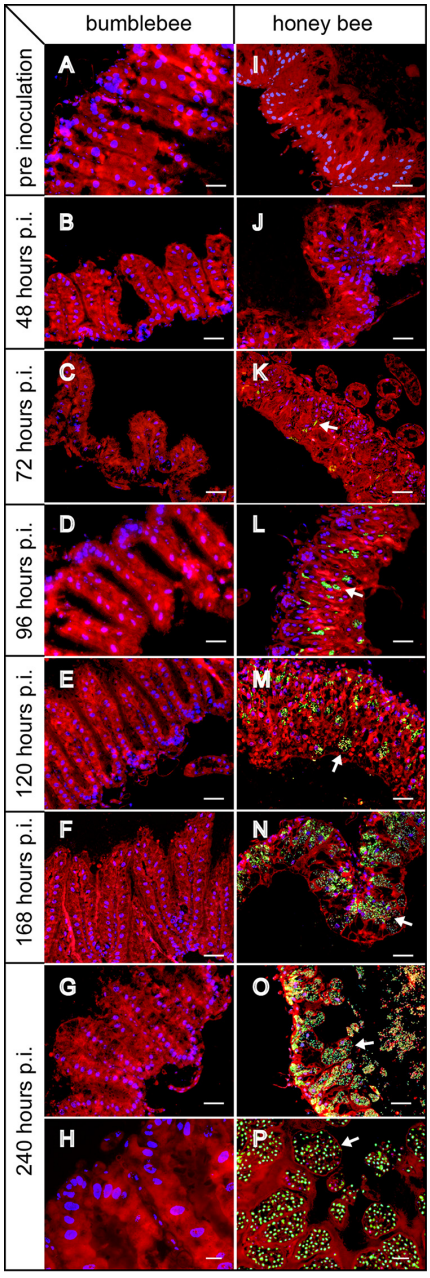


**FIG 4** FISH analysis of midgut sections of experimentally inoculated bumblebees and honey bees. Representative results of *Nosema*-specific fluorescence *in situ* hybridization (FISH) analysis of tissue sections of *B. terrestris* (A to H) and *A. mellifera* (I to P) before inoculation and at 48, 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 $\mu$m (A to G and I to O) and 10 $\mu$m (H and P).

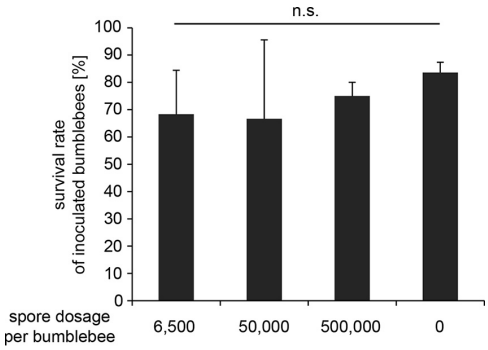


**FIG 5** Survival rates of *B. terrestris* that had been inoculated with different spore dosages. Naive newly hatched bumblebees were orally inoculated with 6,500, 50,000, or 500,000 freshly isolated *N. ceranae* spores. For each spore-inoculated group, experiments were run in triplicate with 20 individuals. 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% ± 0% [mean ± SD]) and mock-inoculated (90% ± 10.0% [mean ± 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-

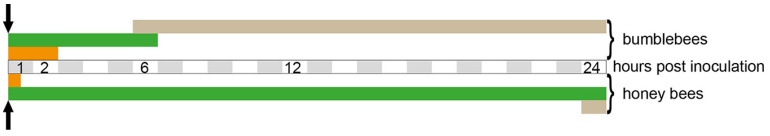


**FIG 6** Passage of *N. ceranae* spores through the intestine of *B. terrestris* and *A. mellifera*. The presence of *N. ceranae* spores in the honey stomach (orange), midgut (green), and hindgut (brown) was determined by microscopic analysis of squash preparations prepared at 30 min, 2 h, 6 h, 12 h, 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 $\mu$l 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 $\mu$l 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 $\mu$l 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-$\mu$l 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-$\mu$l 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 $\mu$m) 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 $\mu$l 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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# 3 Discussion

*Nosema ceranae* is an emergent pathogenic microsporidium in the Western honey bee after it switched host from *A. ceranae* to *A. mellifera* some decades ago. While its congener *N. apis* is considered well researched and of rather low impact on colony health (Bailey & Ball, 1991), the impact of *N. ceranae* infections on *A. mellifera* colonies is controversially discussed. Numerous differences between *N. apis* and *N. ceranae* have been described, e.g. in infection seasonality, in virulence, in dysentery appearance during open infection and in temperature resistance, resulting in its own clinical picture "type C nosemosis" for *N. ceranae* infections (Higes et al., 2010).
In order to address these differences, the publications in this thesis use microscopic and molecular diagnostic methods to determine if an infection is present and which pathogen infects the bee. Microscopic examination can be challenging due to morphological similarities between *Nosema* spp. and the molecular differentiation strongly relies on the used PCR-primers. Considering the variance of sensitivity and specificity in the molecular differentiation of *N. apis* and *N. ceranae* in our studies duplex PCR (Gisder & Genersch, 2013), has been used to avoid biased results in favour of *N. ceranae* or co-infection (Erler et al., 2012; Gisder & Genersch, 2013).
We were able to solve the controversies regarding *N. ceranae* spread, seasonality, dominance, and virulence by analysing the data we obtained in a long-term longitudinal cohort study on honey bee health in Northeast Germany in which data of about 230 individual colonies from about 23 apiaries were continuously collected twice per year during a 15-year period. From this unique and large data set we used a data subsets comprising 5600 spring and autumn bee samples and a data subset comprising 3502 autumn bee samples which allowed for robust and well-found results as presented in Gisder et al. 2017 (attached in 2.1) and Schüler et al. 2022 (attached in 2.2).
We derived seasonal pattern for different *Nosema* infections: the finding of seasonality in *N. apis* infection confirmed accepted knowledge (Fries, 1993), while the finding of the same seasonal pattern in *N. ceranae* infection contradicted existing studies (Higes et al., 2006; Higes et al., 2010). For *N. apis*, infection values are higher in spring than in autumn, which is due to its pathobiology. During winter the *N. apis* infection

is increasing, because many relatively old honey bees (winter bees) are forced to spend long time together in a confined space which facilitates infection of one another. Furthermore, older bees are more easily infected and due to their age simply carry more spores (Meana et al., 2010). They have no chance to defecate outside, resulting in infectious faeces inside the hive which can be ingested orally during cleaning activities. This results in perfect conditions for the spread of the infection inside the hive during the winter or non-flight-season because of the accumulation of infectious material. *N. ceranae* infection values were regularly higher in spring than in autumn for almost the entire study period. In recent studies (Emsen et al., 2020; Porrini et al., 2016) a similar seasonality of *N. ceranae* infection has been observed and could be supported by our long-term study.

Contradicting observations concerning the epidemiology of *N. ceranae* from short-term studies can also be clarified with our long-term data. Numerous publications assume a replacement of *N. apis* by *N. ceranae* due to a decreased number of infections or even a complete disappearance of *N. apis* (Chen et al., 2012; Emsen et al., 2016; Martín-Hernández et al., 2012; Matthijs et al., 2020). As our data shows, the prevalence of *N. ceranae* was significantly increasing in both spring and autumn while *N. apis* infection numbers were constant in spring, but decreased in autumn. Therefore, we concluded that there seems to be competition between *N. apis* and *N. ceranae* during summer, but not during winter. A general replacement of *N. apis* by *N. ceranae* cannot be confirmed by our long-term data. Additionally, as shown in Gisder et al. 2017 (2.1), *N. ceranae* is replicating more effectively at high temperatures than *N. apis*, as may prevail in summer, but its spores are less tolerable towards cold temperatures, as may occur in winter in Northeast Germany.

In regard to the virulence we saw a statistically significant relation between *N. ceranae* infection and winter losses in our study region (Schüler et al. 2022 (2.2)) for the first time. This connection exists exclusively for *N. ceranae* and not for single *N. apis* or co-infections. In our dataset the relationship between *N. ceranae* and winter losses became evident only after 11 years, due to a sufficiently high number of *N. ceranae* infected colonies and therefore, in previous publications from our group, we could not detect the relation between *N. ceranae* and elevated winter losses (Gisder et al., 2010; Gisder et al., 2017).

For low prevalence pathogens a sufficient number of cases is necessary in order to be able to recognize statistically significant connections which otherwise be masked by the effects of high prevalence parasites like *V. destructor*. On the other hand,

statistical significance does not equal biological relevance, as the decision tree and calculation of the biological effect size had shown, assessing the connection as a very small biological effect (Schüler et al. 2022 (2.2)). Our results confirm what has been demanded in science for a long time. Namely, that derivations may not be based solely on statistically significant p-values (Halsey, 2019; Sullivan & Feinn, 2012). The most important variable for explaining colony losses was *V. destructor* infestation, which is known and accepted in science and has been confirmed by our analyses. Thus, the importance of good bee-keeping practices and good *V. destructor* treatment for honey bee health and the survival of colonies is emphasized.

Gisder et al. 2020 (2.3) contributes to the clearance of the host range of *N. ceranae*. Most microsporidia are known to have a narrow host range, nevertheless there are several microsporidia generalists able to invade a broad host range (Wadi & Reinke, 2020). As already mentioned in the introduction the microsporidium *N. ceranae* had been reported to perform interspecies transmission to bumblebees and had been called an emerging pathogen (Graystock et al., 2013; Plischuk et al., 2009) implying the risk for an emerging disease in bumblebees. However, our results do not confirm these findings, but proof that there is no *N. ceranae* infection in bumblebees. It seems that due to the shorter retention time of the pathogens in the midgut, epithelial cells of bumblebees were not infected. In rare cases a bumblebee sample was PCR-positive for *N. ceranae*, but in histological sections using FISH we brought the unambiguous evidence that the epithelial cells of *B. terrestris* are intact and replication of the microsporidia does not take place. However, it must be pointed out that a positive molecular diagnostic test, like a PCR result, is no proof of an infection, as it only proofs the presence of the pathogen's DNA (Brown, 2017). Nevertheless, this method had been used to identify *N. ceranae* "infections" in non-*Apis* hosts or wild bees as reviewed in Grupe and Quandt 2020. Only rarely microscopic examinations are performed which visually proofs the presence of the microsporidia, but still not an infection. A common procedure involves the homogenization of the whole bumblebee (Plischuk et al., 2009) which naturally includes also spores on the surface of the bumblebee, into the detection process even if they are not ingested and therefore unable to infect the host. Also, the macroscopic analysis of the ventriculus, which manifested similar visual features as an infected honey bee (Plischuk et al., 2009), is not a sufficient proof for a *N. ceranae* infection in a new host. Despite the described weaknesses of the method, the scientific community still relies on PCR to proof infections with *N. ceranae* in non-*Apis* hosts (Pislak Ocepek et al., 2021; Plischuk et al., 2021) and consequently,

incorrect interpretations find entrance to scientific books and official publications (McArt, 2021) and even in a comment of the *Central Committee on Biological Safety* (ZKBS) ("Empfehlung der ZKBS zur Risikobewertung von Nosema apis, Nosema ceranae und Nosema bombi als Spender- oder Empfängerorganismen für gentechnische Arbeiten gemäß § 5 Absatz 1 GenTSV", Central Committee on Biological Safety, https://www.zkbs-online.de/ZKBS/SharedDocs/Downloads/01_Allgemeine%20Stellungnahmen/05%20Pilze/Nosema_apis_N._ceranae_N._bombi_2012.html, visited May 19, 2022)(the document refers to Plischuk et al. 2009). In order to prevent wrong conclusions from PCR results, the need for histologic examinations as proof of infection and replication should be anchored in the standards, e.g. COLOSS BEE BOOK and due to the rapid development in research, these standards should be adapted to the current state of knowledge as regularly as possible. To sufficiently proof an infection with *N. ceranae* it is necessary to examine epithelial cells of the midgut to confirm that the spores replicate in the new host as it was done in Gisder et al. 2020 (2.3). The findings were confirmed van der Steen et al. 2022, who show that bumblebees are no true hosts of *N. ceranae* and confirmed it by a novel TaqMan-based RT-qPCR.

In summary, no general advantage of *N. ceranae* over *N. apis* in Northeast Germany could be detected: *N. apis* is neither displaced by *N. ceranae* nor is there evidence of higher virulence. Further, a similar seasonal infection pattern could be observed for *N. apis*, *N. ceranae* and co-infections. Despite a statistically significant connection between *N. ceranae* and colony winter losses, which is only of little biological relevance, *N. ceranae*, at least in our study area do not correlate with the clinical picture of "type C nosemosis" (Higes et al., 2010).

Even if *N. ceranae* is no serious threat to honey bee health under the current circumstances of low prevalence, the monitoring must continue because the prevalence of *N. ceranae* is significantly increasing and the threat to bee health might be higher at a certain point. Especially due to the higher biotic potential of *N. ceranae* in higher temperature shown in Gisder et al. 2017 (2.1) it still needs to be investigated how the climate change may alter the prevalence of *Nosema* spp.

Besides, *N. ceranae* seems to be a microsporidium of narrow host range as we proofed that bumblebees cannot get infected by *N. ceranae* and no convincing interspecies transmission has been published so far. Upcoming reports of interspecies transmission of *N. ceranae* should be viewed with caution unless the germination of *N. ceranae* spores in the new host's midguts and the propagation of *N. ceranae* within the new

host has been shown doubtlessly e.g. by microscopic and FISH analysis.

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# 5 List of publications

## 5.1 Articles

1. Gisder, S., Schüler, V., Horchler, L. L., Groth, D. & Genersch, E. (2017). Long-Term Temporal Trends of *Nosema* spp. Infection Prevalence in Northeast Germany: Continuous Spread of *Nosema ceranae*, an Emerging Pathogen of Honey Bees (*Apis mellifera*), but No General Replacement of *Nosema apis*. Frontiers in Cellular and Infection Microbiology, 7, 301. DOI: 10.3389/fcimb.2017.00301
(Gisder et al., 2017)

2. Gisder, S., Horchler, L. L., Pieper, F., Schüler, V., Šima, P. & Genersch, E. (2020). Rapid Gastrointestinal Passage May Protect *Bombus terrestris* from Becoming a True Host for *Nosema ceranae*. Applied and Environmental Microbiology, 86, 12. DOI: 10.1128/aem.00629-20
(Gisder et al., 2020)

3. Schüler, V., Liu, Y.-C., Gisder, S., Horchler, L., Groth, D. & Genersch, E. (2022). Statistical significance and biological relevance: The case of *Nosema ceranae* and winter colony losses. Preprint deposited on bioRxiv.org. DOI: 10.1101/2022.05.20.492825
(Schüler et al., 2022)

## 5.2 Poster and Talks

1. Poster: $64^{th}$ Annual Meeting of the Institutes for Bee Research e.V., Celle, 14. - 16. March 2017, Pieper, F., Gisder, S., Schüler, V., Horchler, L.; Sima, P. & Genersch, E.: Experimentelle Infektion von gekäfigten Honigbienen und Hummeln mit *Nosema ceranae*

2. Poster: $69^{th}$ Annual Meeting of the Institutes for Bee Research e.V., organized in Hohenheim, 05. – 07. April 2022, Schüler, V., Gisder, S., Liu, Y.-C., Groth, D. &

Genersch, E.: Sind Winterverluste in Honigbienenvölkern in Nordostdeutschland aufgrund von *Nosema ceranae* Infektionen erhöht?

3. Talk: Grako 2046 annual retreat, Berlin, 2020, Schüler, V.: Honey bee colony winter losses in Northeast Germany: is there a connection to *Nosema ceranae* infections?

4. Talk: Grako 2046 annual retreat, Berlin, 2021, Schüler, V.: Rapid Gastrointestinal Passage May Protect *Bombus terrestris* from Becoming a True Host for *Nosema ceranae*