Eesti Maaülikool
Estonian University of Life Sciences

FACTORS AFFECTING BEEKEEPING SUSTAINABILITY: PATHOGEN SPREAD, DIAGNOSTICS AND QUEEN BREEDING

MESINDUSE JÄTKUSUUTLIKKUST MÕJUTAVAD FAKTORID: PATOGEENIDE LEVIK, DIAGNOSTIKA JA MESILASEMADE ARETUS

SIGMAR NAUDI

A Thesis
for applying for the degree of Doctor of Philosophy
in Agricultural Sciences

Väitekiri
filosoofiadoktori kraadi taotlemiseks
põllumajanduse erialal

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LIST OF ORIGINAL PUBLICATIONS

This thesis based on the following papers, which are referred to by their Roman numerals in the text. All papers are reproduced with the kind permission of the publishers.

I. **Naudi, S.**, Šteiselis, J., Jürison, M., Raimets, R., Tummeleht, L., Praakle, K., Raie, A., Karise, R. (2021). Variation in the distribution of *Nosema* species in honey bees (*Apis mellifera* Linnaeus) between the neighboring countries Estonia and Latvia. Veterinary Sciences, 8 (4):58. DOI: 10.3390/vetsci8040058.

II. **Naudi, S.**, Rossi, F., Ricchiuti, L., Manocchio, P., Del Matto, I., Jürison, M., Viinalass, H., Karise, R., Raimets, R. (2025). Could hive debris samples and qPCR ease the investigation of factors influencing *Paenibacillus larvae* spore loads? Journal of Apicultural Research, 1–10. DOI: 10.1080/00218839.2024.2435222

III. Raimets, R., **Naudi, S.**, Bartkevics, V., Smaggeh, G., Mänd, M., Karise, R. (2019). Translocation of tebuconazole between bee matrices and its potential threat on honey bee (*Apis mellifera* Linnaeus) queens. Insects, 13(1):45. DOI: 10.3390/insects13010045

IV. **Naudi, S.**, Raimets, R., Jürison, M., S. Liiskmann, E., Mänd, M., Salkova, D., Karise, R. (2022). Effect of *Nosema apis* and *N. ceranae* on honey bee *Apis mellifera* queen development. Acta Morphologica et Anthropologica, 29 (3-4), 178-182. DOI: 10.7546/AMA.29.3-4.2022.33

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Paper	I	II	III	IV
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Data analyses and interpretation	**NS**, ŠJ, JM, KR	**NS,** RF, RL, MP, DMI	RR, KR, BV, MM; SG	NS, KR
Manuscript preparation	**NS**	**NS,** RR, KR	**NS,** RR, KR	NS
Proofreading and commenting	All authors	All authors	All authors	All authors

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ABBREVIATIONS

CCD	Colony Collaps Disorder
AFB	American Foulbrood
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
HQ	Hazard Quotient
LD_{50}	Median Lethal Dose
CFU	Colony Forming Units
UHPLC-MS/MS	Ultra-High-Performance Liquid Chromatography coupled with Tandem Mass Spectrometry
BQCV	Black Queen Cell Virus
DNA	Deoxyribonucleic acid

1. INTRODUCTION

The honey bees (*Apis mellifera* Linnaeus, 1758) are in unique situation compared to other pollinators – beside few wild populations (Kohl & Rutschmann, 2018) most of the colonies are actively managed. Recently, the number of managed honey bee colonies has begun to increase again (Phiri *et al.*, 2022). The level of management effort and beekeeper interest depends on both economic and natural factors. Economic factors include the state of the honey market, honey prices, and competition with fake honey (Ždiniaková *et al.*, 2023). Natural factors encompass the spread of diseases and parasites, poor nutrition due to changes in floral availability (Naudi, 2023; Van Espen *et al.*, 2023), pesticide exposure (Woodcock *et al.*, 2017), habitat loss (Regan *et al.*, 2015; Sonmez Oskay *et al.*, 2023), climate change (Belsky & Joshi, 2019; Neov *et al.*, 2019; Pasquale *et al.*, 2016), and combinations of these challenges.

One of the most significant challenges in beekeeping is the limited success of honey bees in overwintering, often influenced by disease management practices (Maes *et al.*, 2021). The most prevalent disease affecting honey bees is varroosis, caused by the *Varroa* mite (*Varroa destructor* Anderson and Trueman, 2000). This parasite also serves as a vector for transmitting viruses (Warner *et al.*, 2024) and has garnered substantial scientific attention in recent years. However, other common diseases, such as nosemosis (Iorizzo *et al.*, 2022; Mazur & Gajda, 2022) and American foulbrood (AFB) (Hansen & Brødsgaard, 1999), present significant detection and management challenges.

Nosemosis is caused by microsporidian pathogens *Nosema apis* Zander, 1909 (Fries, 1993) and *N. ceranae* Fries, 1996 (Fries *et al.*, 1996). These species differ in severity and disease progression, but both are difficult to diagnose due to non-specific symptoms. For example, symptoms like diarrhea may be mistaken for defecation caused by starvation or other stressors (Hristov *et al.*, 2020).

Another widespread pathogen is American foulbrood, caused by *Paenibacillus larvae* White, 1906 (Morrissey *et al.*, 2015). Although its clinical signs are distinct, eradicating it remains problematic (Matović *et al.*, 2023). In the European Union, infected colonies must be destroyed, and all equipment thoroughly disinfected (Regulation (EU) 2016/429).

While modern DNA-based techniques can detect low spore levels, these do not necessarily indicate an impending outbreak, complicating the decision on when to intervene (Stephan *et al.*, 2020). As a result, these techniques, though valuable for early detection, add complexity to management decisions by leaving uncertainty about the spore threshold at which action is required.

The success of a honey bee colony also relies on the health and quality of its queen bee, an area less studied due to experimental difficulties. Despite the significant protection offered by the colony and the queen's unique physiological traits, she remains vulnerable to certain diseases and environmental factors. Infections can be transmitted through the eggs she lays, potentially affecting the entire colony (Abou-Shaara *et al.*, 2021). For example, infection with *Nosema* species in the queen can lead to the development of Black Queen Cell Virus (Gajda *et al.*, 2021). If the queen is infected with pathogens or of poor quality (e.g., not laying, or producing an aggressive colony), she may be replaced by worker bees or the beekeeper. This is particularly common when her physiological traits and pheromone production are disrupted (Alaux *et al.*, 2011).

Raising high-quality queen bees is a complex and time-consuming process requiring significant expertise. Many beekeepers choose to purchase queens from specialized producers or import them internationally (Bieńkowska *et al.*, 2020; Bixby *et al.*, 2020). However, this approach carries risks, as queens from large-scale breeders may exhibit weaker traits due to high production volumes or may not adapt well to local conditions (Holmes *et al.*, 2023). Beekeepers also suspect that these queens are not routinely tested for diseases, apart from American foulbrood, small hive beetle, and *Tropilaelaps* mite, as required by the EU Delegated Regulation (EU) 2020/692. This lack of routine disease testing increases the potential for disease transmission.

This PhD thesis focuses on the challenges associated with the spread and diagnosis of bee diseases, as well as the role of beekeepers' choices in honey bee colony reproduction.

2. REVIEW OF THE LITERATURE

Honey bees are classified within the phylum Arthropoda, belonging to the class Insecta and order Hymenoptera, specifically within the family Apidae and genus *Apis*. Currently, the genus *Apis* is known to comprise eight distinct species, notably including the Western honey bee (*Apis mellifera*) and the Asian honey bee (*A. cerana* Fabricius, 1793), which are among the most recognized. The Western honey bee is being considered one of the most important pollinators of agricultural as well as wild plants and can be found all over the world (Moreno, 2016). In colder climate conditions, the honey bee persistence depends on human activity to guarantee overwintering (Southwick, 1991). Beekeeping practices are variable and similarly to domestication of any animals, aggregating bees into larger groups promotes the spread of diseases or pose the bees to other human-driven stressors.

2.1. Changes in the number of honey bees over time

Honey has been a valuable source of sweet nutrients for thousands of years. Evidence of its historical use includes a cave painting found in Spain, depicting honey gathering, which is estimated to be around eight thousand years old (Kritsky, 2017; Prendergast *et al.*, 2021). Today, honey bees are globally spread, managed by humans for commercial beekeeping products and to support pollination, both contributing to our food security (Jongh *et al.*, 2022).

Modern beekeeping, which took shape in the 18th and 19th centuries, saw the development of human-made hives through a series of experiments. Remarkably, contemporary beekeeping practices remain akin to those of the 19th century. Surprisingly, many contemporary beekeeping practices have remained relatively unchanged since then. During this time, the number of honey bee colonies worldwide has grown substantially. Currently, the Food and Agriculture Organization estimates that there are approximately 102 million honey bee colonies globally, with Asia hosting the largest share at 45.3 million colonies, followed by Europe with 25.1 million colonies (FAO, 2023).

Over several decades, the population of honey bees has been in constant fluctuation (Osterman *et al.*, 2021; Phiri *et al.*, 2022; Potts *et al.*, 2010;

vanEngelsdorp & Meixner, 2010). Short-term sharp declines in bee populations have occurred repeatedly. One example is the incident on the Isle of Wight around 1910, where there was a gradual die-off of honey bees (Bailey, 1964), the cause of it is still unknown (Underwood & VanEngelsdorp, 2007). Another example is the case between 2007 to 2010 in the USA, where the honey bee colonies just vanished before the wintering. This occurrence was very abrupt and widespread throughout North America (the US and Canada), leading to a drastic decrease in the number of honey bee colonies (vanEngelsdorp & Meixner, 2010). The cause of this colony collapse syndrome is not fully understood, and it continues to be referred to by the broad term CCD (Colony Collapse Disorder). Since the year CCD emerged, mortality rates in beekeeping have drastically increased from earlier 10–20% (VanEngelsdorp *et al.*, 2007) to average of 30–60% (Steinhauer *et al.*, 2023). In Europe, honey bee colony mortality has also emerged as a major concern in recent decades. Current winter losses of bee colonies typically range between 10% and 30% (Clair *et al.*, 2022; Gray *et al.*, 2020). Research indicates that this rising mortality rate may be partly linked CCD in Europe as well (Dainat *et al.*, 2012).

The high mortality rate of honey bees results from a complex interaction of abiotic, biotic, and beekeeping management factors, with management practices often playing a particularly critical role. Throughout history, humans have significantly shaped and influenced wild animal populations, including honey bees. The development of modern beekeeping has unintentionally facilitated the spread of parasites and pathogens within honey bee colonies, a problem further intensified by global trade and the effects of globalization (Brosi *et al.*, 2017). Additionally, abiotic factors such as habitat loss and climate change further exacerbate these challenges (Jongh *et al.*, 2022).

Despite the high annual mortality rates, the global number of honey bee colonies has shown an overall growth trend. For example, between 2000 and 2017, the number of honey bee colonies in North America increased by 5%, while Europe saw a rise of 23.2% (Phiri *et al.*, 2022). Compared to 1990, the number of colonies in Europe has grown by 11.6% (Figure 1). Similarly, North America experienced an estimated 20% increase, and Asia saw an even more dramatic rise of approximately 95%, with colony numbers growing from 23.1 million in 1990 to around 45.3 million today (Phiri *et al.*, 2022).

This growth has been primarily driven by rising honey consumption and the increasing demand for beekeeping products, including pollination services. However, establishing new colonies comes at a cost. Low breeding success can make the process expensive and may lead to intensified competition among beekeepers and honey bee populations, as well as between colonies themselves (Abrol, 2013; Jaatinen, 2024).

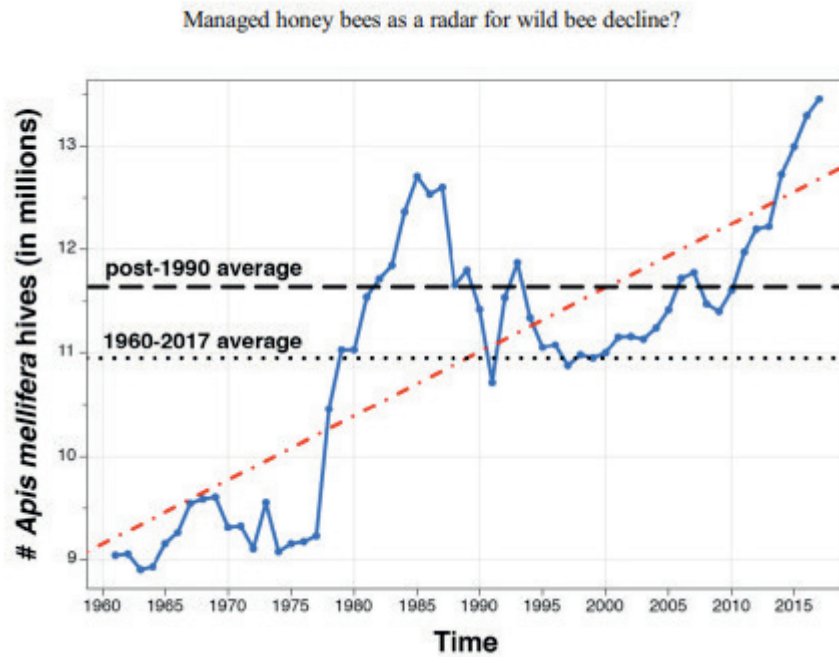

Figure 1. The number of managed honey bee colonies in Europe between 1961 and 2017 (data from FAO (Phiri *et al.*, 2022)).

2.2. Biotic factors affecting beekeeping: diseases and parasites

Honey bees are social insects, akin to a single superorganism with thousands of inviduals within a colony, a characteristic facilitating the spread of diseases within the colony. The high level of social interaction and cooperation necessary for colony survival means also that pathogens can easily spread from one individual to another, making the entire colony vulnerable to infections (Page *et al.*, 2016). Within the colony, the bees rub against each other, also trophallaxis - oral sharing of food - occurs in the colony and supports the spread of diseases. The main cause of honey bee losses are parasites and pathogens such as the *V. destructor* and *Nosema* sp. (Neov *et al.*, 2019; Higes *et al.*, 2007). Until the 1970s,

brood diseases like AFB (Matović *et al.*, 2023) were the main problem for beekeepers and honey bees and thereafter *Varroa* mite and nosemosis have become the dominant threats (Ii & Quandt, 2020; Morfin *et al.*, 2023).

The variety of pathogens and parasites affecting honey bees is extensive, with AFB being particularly prevalent all over the world (Matović *et al.*, 2023). However, some colonies have shown higher resistance due to increased hygienic behavior. This natural behavior is crucial for maintaining colony health, as it involves detecting and removing infected or dead larvae from the hive, thereby reducing the spread of disease (Bigio *et al.*, 2013). This hygienic trait is genetically inherited and can be passed down through queen bees when controlled breeding is practiced by beekeepers (Sprau *et al.*, 2023). Although selecting for high hygienic behavior is a relatively new strategy in disease management, it shows promise for mitigating other stressors in the future. However, widespread adoption has been slow, as testing for hygienic behavior requires specialized skills from both queen breeders and beekeepers (Arbia & Babbay, 2010; Facchini *et al.*, 2019; Gerdts *et al.*, 2018).

The virulence of a pathogen is influenced by its spreading mechanisms. For example, highly virulent bacterial diseases may rely on persistent spores to facilitate their spread even after the death of the colony (Fries & Camazine, 2001). virulence and pathogenicity are distinct, and pathogens with varying modes of transmission can exhibit high virulence. The less virulent pathogens do not kill the colony easily and are transmitted by contacts of bees from different colonies, at the same time, there is less need for persistent spores (Fries & Camazine, 2001).

Strict control measures employed to manage AFB have proven effective in eradicating the disease in certain regions (Stephan *et al.*, 2020). Long-term observations from England and Wales indicate that high-incidence clusters of AFB do not persist in the same regions, suggesting the possibility of eradicating the disease from those areas (Mill *et al.*, 2014). Furthermore, the authors identify both endemic and exotic risk factors for the disease.

Nosemosis, tends to be a more persistent issue in certain regions, as observed in both England and Spain, where *N. ceranae* incidents have increased over time (Botías *et al.*, 2012). Moreover, research by Kurilov

et al. (2020) and Schüler *et al*. (2023) has drawn connections between *Nosema* infections and CCD, noting an increase in *Nosema* infections associated with CCD cases.

Honey bee parasites can also have variable impacts on colonies. Similar to low-virulence pathogens, low-impact parasites spread via direct contact of honey bee individuals. Various mites (*Varroa* mite, *Tropilaelaps clareae* Delfinado & Baker, 1962, and *Acarapis woodi* Rennie, 1921) spread through contact between bees from different colonies, either on flowers or during robbing activities in hives (De Jong *et al*., 1982; Koeniger & Muzaffar, 1988; Sammataro *et al*., 2000). The small hive beetle (*Aethina tumida* Coleoptera: Nitidulidae, Murray, 1867), is rapidily killing the colony and spreads via beetles active searching of new host or is spread by human activity. It may also be transported through horticultural products that use soil where the beetle pupates (e.g., flower pots) or through fruit trading (Neumann *et al*., 2016; Stief *et al*., 2020). A new threat, the Asian hornet (*Vespa velutina* Lepeletier, 1863), is an invasive species that is rapidly spreading across Europe due to its large suitable temperature range for habitat expansion (Barbet-Massin *et al*., 2020; Dillane *et al*., 2022).

To mitigate these threats, European countries need robust regulations to prevent the spread of honey bee parasites (Kennedy & Osborne, 2023).

2.2.1. Microsporids *Nosema ceranae* and *N. apis*

Microsporidian pathogens from the genus *Nosema* sp. (also classified as *Vairimorpha* sp. by Tokarev *et al*., 2020, though this classification is questioned by Bartolomè *et al*. (2024) cause a disease in honey bees called nosemosis. *Nosema apis* was the initial microsporidian species identified in honey bees (Fries, 1993). Another species, *N. ceranae*, was initially described in *A. cerana* in China in 1996 (Fries *et al*., 1996). It appears that *N. ceranae* has crossed the species barrier and infested *A. mellifera* in Taiwan in 2005 (Huang *et al*., 2007). There is evidence of *N. ceranae* detection in Europe since the mid-1990s (Paxton *et al*., 2007).

Although the two *Nosema* species are considered similar, they exhibit distinct patterns in honey bee infections. *N. apis* infection peaks occur in autumn and spring, with lower levels during the hot summer months (Botías *et al*., 2013). This aligns with the abundance of older forager

bees in the hive, as they are more susceptible to the pathogen. *N. ceranae* infections may peak in different times through the season (Martín-Hernández *et al*., 2018), but can show also no seasonality (Martín-Hernández *et al*., 2012). The work of Emsen *et al*. (2020) suggests that the seasonality of *N. ceranae* may be directly linked to the honey bee life cycle. The spores of both pathogens reflect a similar development pattern. When they begin to develop in the midgut, this process is relatively similar, and they reach their peak population around 10-12 days after infection (Paxton *et al*., 2007). *N. ceranae* is considered more virulent to the worker bees (Forsgren & Fries, 2010) affecting the viability of honey bees (Gajda *et al*., 2021; Pent *et al*., 2023).

These two pathogen species can also co-exist in the same beehive. Since first description of nosemosis, the *N. apis* was prevailing, but was gradually replaced by *N. ceranae*. Today, *N. ceranae* has effectively displaced *N. apis* in many regions (Burnham, 2019; Holt & Grozinger, 2016). Its ability to thrive in diverse climatic conditions has led to varying prevalence rates worldwide (Martín-Hernández *et al*., 2018). Only a few countries, for instance Mexico, have reported that the dominant species is *N. apis* (González *et al*., 2020). Studies have shown that when interacting with *N. apis*, *N. ceranae* is capable of displacing the latter (Emsen *et al*., 2016; Holt *et al*., 2013; Holt & Grozinger, 2016; Martín-Hernández *et al*., 2012).

Globalization, open borders, and the international transport of queen bees and colonies with minimal control have significantly increased the risk of spreading various diseases, such as *Nosema* (Mutinelli, 2011; Smith *et al*., 2013). This disease, can easily be transmitted through infected queens and colonies. Therefore, it is crucial to replace infected queens with healthy ones within one month since the detection to effectively mitigate the spread of nosemosis (Czekońska, 2000). As the diseased queens experience a decrease in ovarian development (Alaux *et al*., 2011), they have altered level of vitellogenin, reduced life-span and impared queen mandibular pheromone production, which results in failure to control the worker bees (Moritz *et al*., 2002; Vergoz *et al*., 2009).

The European Union has prohibited most commonly used treatments for nosemosis. Currently, no effective medications exist to combat the disease. Natural remedies like Thymol are promoted, but their effectiveness is questionable (Burnham, 2019; Glavinic *et al*., 2022).

Even if they were to work, they need to be used at very specific times when the environmental conditions are suitable for nosemosis control. The only antibiotic medicament for *Nosema* treatment is Fumagilin–B, but it is banned in the European Union (Regulation (EU) 2016/429). Additionally, various nutraceutical and immuno-stimulatory, seemingly natural compounds have been tested, but they still require further research today (Di Sotto *et al.*, 2020).

2.2.2. Bacterium *Paenibacillus larvae*

American foulbrood is a bacterial infectious disease caused by *Paenibacillus larvae*, which primarily affects honey bee brood. This spore-forming, flagellated bacterium is highly resistant to environmental conditions (Jończyk-Matysiak *et al.*, 2020). The disease was first identified in 1903 by George F. White in Europe and has since become a widespread issue across the globe (Genersch, 2010). While the bacterium itself is vulnerable to environmental factors and disinfectants (Tlak Gajger, Tomljanović, *et al.*, 2024), the spores it produces are highly resistant to unfavorable conditions and are released to the environment following the death of the bee larva (Matović *et al.*, 2023). These spores are the primary challenge in controlling and preventing AFB, as they can survive in honey, on brood frames, or on beekeeping equipment for over 50 years, withstanding temperatures of up to 150 °C (Calesnick & White, 1952).

In a bee colony, the infection is spread by young nurse bees, which are responsible for cleaning the hive and feeding the larvae. The larvae, particularly those up to 36 hours old, are susceptible to infection when they ingest food contaminated with spores (Genersch, 2010). Once inside the larva's intestine, the spores develop into vegetative bacteria that multiply rapidly, overwhelming the larva's immune system. Six to seven days after infection, the larvae die (Hansen & Brødsgaard, 1999). The dead larvae, found in capped cells, turn from grayish-white to brown and eventually become a sticky, foul-smelling mass. This characteristic appearance of dead larvae in combs is a key indicator of AFB (Matović *et al.*, 2023).

While adult bees cannot become infected with AFB, they can carry and transmit spores to other colonies, especially through robbing behaviors and contact with nearby hives. Colonies within a 2-3 km radius are at a

high risk of infection (Datta *et al.*, 2013; Mill *et al.*, 2014). Beekeepers may also spread the disease unintentionally through contaminated tools and clothing, making strict disinfection protocols essential when AFB is detected in a colony (Piccolomini, 2024). Given the high transmissibility of spores and the inevitable colony death once symptoms appear, many countries enforce strict control measures. The most effective, though costly, method is to burn the entire infected colony, including the honey frames, to prevent further spread. For example, in Canada, this is a legal requirement (Ontario Beekeepers Association, 2012).

In Estonia, the detection and treatment of AFB adhere to the EU regulations outlined in Regulation (EU) 2016/429. Some other methods are also used for controlling AFB. For colonies showing milder symptoms, such as uneven brood, faint odor, and discolored larvae, a technique known as shook swarm is employed (Matović *et al.*, 2023; Mosca *et al.*, 2023). This method involves removing all adult bees from the affected hive and transferring them to clean equipment. The purpose is not to remove infected bees, but rather to eliminate the disease-causing agents from the hive environment. The old brood and contaminated equipment are destroyed, typically by burning, to prevent the spread of the disease. The adult bees are then introduced to a new, clean hive, significantly reducing the likelihood of disease transmission (Waite *et al.*, 2003). Shook swarm is thought to be the most effective treatment method. While it helps reducing the number of *P. larvae* spores to a minimum, it doesn't completely eliminate spores but rather alleviates the level of infection within the bee colony (Pernal *et al.*, 2008).

Antibiotic treatments, including oxytetracycline and tylosin, have proven effective against AFB (Abdel-Baset *et al.*, 2019). However, their use has been banned in the European Union since 2006 due to concerns about promoting antibiotic resistance in *P. larvae* (Masood *et al.*, 2022). Moreover, the presence of antibiotic residues in bee products poses significant risks to human health by contributing to antibiotic resistance (Okocha *et al.*, 2018).

Current AFB regulations in Estonia lack clear guidance regarding the destruction of infected colonies, and there is no compensation mechanism for beekeepers who lose their colonies due to the disease (Regulation (EU) 2016/429). However, this situation varies by country, as some nations do offer compensation for such losses (Agriculture Victoria,

2024; American Foulbrood Management Agency, 2024). For example, in Europe, Germany provides compensation mechanisms for beekeepers through the Animal Disease Fund (Tierseuchenkasse) (Ministerium für Energiewende, 2024).

The definition of AFB is not unequivocally clear – whether the presence of clinical symptoms should be considered as threshold to decide to burn the colony, or whether finding even a single spore, which using DNA based methods is very likely, should be followed by the strict safety measures (Gende *et al.*, 2011; Hansen & Brødsgaard, 1999). This divergence in interpretation is significant because modern techniques can easily detect minimal spore levels, raising the question of when strict biosecurity protocols, such as colony destruction or quarantine, should be applied.

2.3. Management issues affecting sustainable beekeeping

Beekeeping is a sector that faces numerous challenges, both from internal management practices and external factors such as agricultural activities, climate change, and environmental pollution (Barahona *et al.*, 2024). Beekeepers directly influence the health of their bees through their management decisions, including the use of mite control treatments that contain synthetic pesticides. When misused, these treatments can lead to increased bee mortality (Bahreini *et al.*, 2020; Mancuso *et al.*, 2020).

On the other hand, agricultural producers can have a positive impact on honey bees by creating new foraging areas, cultivating cover crops, and enhancing foraging potential through the growth of entomophilous crops (Rands & Whitney, 2011; Vaughan *et al.*, 2015). This is especially important in northern climates, where the number of natural plants with overlapping flowering periods is limited, and they do not form large blooming areas (Goulson, 2009). By growing crops that provide foraging resources, agricultural producers can ensure that bees have access to abundant food, which benefits beekeepers and their harvests (Karbassioon *et al.*, 2023; Leonhardt *et al.*, 2013; Malagnini *et al.*, 2022). However, agricultural practices can also negatively affect bees by introducing pesticides into bee products and directly harming the bees themselves (Laurent *et al.*, 2024; Raimets *et al.*, 2019). Despite substantial efforts in the last decade, there is little evidence that Europe has achieved the reduction in pesticide risks and impacts as mandated in

National Action Plans (Lee *et al.*, 2019; Möhring *et al.*, 2020). Which still leaves the nature of the problem quite common and relevant.

To ensure sustainable and economically viable beekeeping, maintaining the health of bee colonies and individual bees is essential (Honey Bee Health Coalition, 2019). Stress factors related to management practices - such as exposure to pesticides, diseases, and parasites - have a significant impact on bee health (Dickey *et al.*, 2023; Vanbergen, 2021).

The health of the queen bee, in particular, is critical for colony success. Stressors like pesticides can impair the queen's fecundity, affecting her ability to lay eggs (Wu-Smart & Spivak, 2016) or alter her behavior (Williams *et al.*, 2015) which in turn can compromise the overall health and productivity of the colony (Tokach *et al.*, 2024).

2.3.1. Pesticides induced stress

In the context of intensive agriculture, plant protection products are widely used. Until 2014, one of the primary concerns was nicotine-like neuroactive organic substances known as neonicotinoids (Lundin *et al.*, 2015). Neonicotinoids adversely affect honey bees by impairing their orientation abilities, weakening immune system, memory, and inducing behavioral changes that reduce foraging efficiency (Belzunces *et al.*, 2012). These substances also cause physiological changes and reduce the longevity of worker bees (He *et al.*, 2021). In present, the concern extends beyond neonicotinoids to include other classes of insecticides (Urlacher *et al.*, 2016), but also fungicides (Reiß *et al.*, 2023) and herbicides (Wang *et al.*, 2022) either as single compounds or in mixtures (Gómez-Méndez *et al.*, 2023; Raimets *et al.*, 2019). Moreover, agricultural pesticides interact with apicultural medicinal products (Johnson *et al.*, 2013) and cause subsequent health problems to honey bees (Garrido *et al.*, 2016).

While sublethal concentrations of pesticides may seem relatively mild in their impact, their effects can be significantly amplified when combined with other stress factors. Malnutrition and the presence of concurrent diseases exacerbate the harmful effects of pesticides, making these stressors increasingly difficult for bee colonies to withstand (Almasri *et al.*, 2021; Monchanin *et al.*, 2019).

2.3.2. Queen rearing and dependence on stress factors

The general practice in beekeeping is to replace queen bees either annually or every two years, as their egg-laying activity can decline by up to 15% or more after the second year compared to one-year-old queens (Hauser & Lensky, 1994), which in turn leads to a reduction in honey productivity. The productive lifespan of queen bees typically ranges from 2 to 3 years, and it is rare for them to maintain high quality beyond the third year. However, their genetic traits remain intact, allowing them to be used for breeding purposes if necessary (Remolina & Hughes, 2008).

Naturally, worker bees are responsible for rearing new queens. First, they prepare the brood cell (clean it and, if necessary, polish it to the right size). Then, the current queen bee in the hive lays an egg in this brood cell. The embryonic stage for the queen bee lasts three days, during which the egg develops into a larva. During this time, nurse bees place royal jelly near the egg.

The larval stage lasts from the 3rd to the 8th day. On the 7th day, the queen cell is sealed, marking the beginning of the pupal stage, which lasts approximately 7 - 8 days. During this period, the larva undergoes metamorphosis. In total, the development of a queen bee from egg to adult takes about 16 days (The Polyandrous Queen Honey Bee, 2003).

Artificial queen breeding is a very complex and precision-demanding activity in order to achieve maximum quality. The success of queen bee rearing can depend on many factors (Gemedi & Abi, 2023; Büchler *et al.*, 2023). Beekeepers generally aim for a high or very high grafting efficiency, which means that the acceptance rate of queen cells by worker bees in nursing colony should be at least 80% (Cengiz *et al.*, 2019; Okuyan & Akyol, 2018). Either biotic and abiotic factors can also affect the acceptance rate of grafted queen cells and subsequent history the developing queens. For instance, the foraging conditions for the nursing colony must be favorable, and a substantial amount of fresh nectar must be brought into the hive (Gencer *et al.*, 2017; Hussain *et al.*, 2020), otherwise the bees have no stimuli to feed the larvae (Cengiz *et al.*, 2019).

Since queen cell building colonies generally consist of young nurse bees who do not perform foraging flights, these colonies are often fed sucrose

syrup (dilution of 1:1 w/w) to mimic the nutritional properties of natural nectar and to enhance grafting efficiency (Gençer *et al.*, 2000).

Furthermore, factors such as the location of the frame within the starter colony can influence the success of grafting. Additionally, the condition of the artificial queen cup, whether it is already moist with royal jelly or dry before the larva is grafted, plays a role (Cengiz *et al.*, 2019; The Polyandrous Queen Honey Bee, 2003).

The season during which the grafting occurs also matters; grafting is generally more effective during peak nectar flow seasons (Lee *et al.*, 2017).

Various stress factors can also influence queen bee development. One of the most serious threats is the Black Queen Cell Virus (BQCV), which targets queen bees during the pupal stage, halting development and causing the pupae to turn black (Tapaszti *et al.*, 2009). Additionally, pesticide residues in wax and food can negatively impact queen development, although they are less significant compared to BQCV (DeGrandi-Hoffman & Chen, 2017).

3. AIMS AND HYPHOTHESIS OF THE STUDY

Modern beekeeping faces ongoing challenges, including pests, diseases, habitat loss, and environmental stressors, requiring continuous management and adaptation. For beekeeping to be sustainable, it is crucial, that the bees remain healthy and beekeepers possess professional knowledge of the risk factors that can reduce sustainability.

Some honey bee diseases are not easily detectable without laboratory testing or can hide the symptoms for long. Conventional pathogen detection methods do not allow extensive monitoring or assessment the presence of pathogens in non-symptomatic colonies (Cameron *et al.*, 2021). There is shortage of knowledge about factors affecting the persistance of the diseases over time and in distribution in spatial scale (Mill *et al.*, 2014).

Beyond colony management, there is an acute deficiency on the knowledge about factors affecting requeening of colonies. Requeening is a process with large natural losses and any additional stress factor affects it even more (Büchler *et al.*, 2023). Moreover, the factors limiting mating success are not well understood (Smilga-Spalvina *et al.*, 2024).

To address these gaps, the following aims and hyphothesis have been set:

1. To assess previously *Nosema*-positive apiaries from 2013, to evaluate the persistence of the disease, and to determine the distribution of both *N. apis* and *N. ceranae* in Estonia and Latvia (**Paper I**).

   H1.1: The distribution and prevalence of *Nosema* species are changing, resulting in the dominance of *N. ceranae* over *N. apis.*
   H1.2: Beekeepers cannot rid their colonies of *Nosema* even when they know that the hives had been infected, as the disease is very persistent.

2. To estimate the distribution and levels of *P. larvae* spore contamination in Estonian apiaries and to define the factors affecting the spread of this pathogen **(Paper II)**.

H2.1: Utilizing hive debris for pathogen *P. larvae* detection, especially with qPCR technology, could serve as a predictive tool for assessing colony health and early detection of AFB.
H2.2: The most important factor in spread of *P. larvae* spore in Estonian apiaries is honey bee density with specific apiary characteristics such as apiary size, abundance of and distance to other apiaries.

3. To study whether and how the fat-soluble fungicide tebuconazole is translocating through different bee hive matrixes (**Paper III)**.

   H3.1: Contaminants like tebuconazole can translocate between different materials within the hive.
   H3.2: Despite the queen bee's primary diet of royal jelly, which is less likely to be contaminated, the constant exposure to potentially contaminated wax combs could pose a risk to her health.

4. To understand, how the presence of spores of *N. apis, N. ceranae* and the mix of both in queen`s royal jelly affects the development of the queen bee from egg to maturity (**Paper IV**).

   H4.1: The pathogen spores in the royal jelly affects the development of the queen bee.
   H4.2: The pathogen spores reach the mature queen bee by eating infected royal jelly.

4. MATERIALS AND METHODS

4.1. Field studies

4.1.1. Apiaries

4.1.1.1. *Nosema* sp. (I)

To understand the persistence of nosemosis in apiaries the study was undertaken in Estonia (2017) and Latvia (2018).

We focused on apiaries that had previously tested positive for nosemosis during the EPILOBEE study (2012–2014) (Jacques *et al.*, 2017). In Estonia, a total of 30 and in Latvia, 60 apiaries were included in our study.

4.1.1.2. *Paenibacillus larvae* (II)

In the spring of 2022, we conducted a study to obtain a comprehensive overview of the spread of *Paenibacillus larvae* spores across Estonia. To ensure adequate coverage across the Estonia, all county beekeeping advisors were invited to participate. The study was relying on the voluntary participation guaranteeing no pre-defined selection of the apiaries.

4.2. Sample collection

4.2.1. *Nosema* sp. (I)

To maximize the likelihood of detecting spores per bee, samples were gathered in May, right before the seasonal transition from winter to summer bees. In each sampled apiary, we gathered about 60-100 forager bees from 2 to 4 adjacent hives using a portable vacuum until enough bees was gathered. A new nylon stocking was placed on the vacuum cleaner nozzle, which was changed after each sample to prevent cross-contamination. These samples were immediately stored in plastic tubes,

chilled for transport, and kept frozen at -20 °C until they could be analyzed in the lab.

4.2.2. *Paenibacillus larvae* (II)

Local county inspectors were responsible for collecting hive debris samples. they had been previously trained with specific protocols to ensure accurate and consistent sample collection. The study took place during the period when beekeepers typically replace hive bottoms to mitigate pathogen transmission and lower pathogen levels. To mitigate the risk of cross-contamination between different bee colonies and apiaries, inspectors were equipped with disposable supplies, including 1500 ml sample containers, nitrile gloves, disposable overalls, and plastic shoe covers.

The sample collection for this study consisted of two distinct methodologies: pooled or individual hive sampling. In pooled sampling, each of these was a composite from 2 to 10 hives. In cases where the apiary housed more than 10 hives, additional pooled samples were gathered. For comparison purposes, individual hive samples were collected from three apiaries in total of 42 samples.

Approximately 500 grams of a mixture of dead bees and wax debris were collected for the study at all sampling methods. These samples were immediately frozen post-collection and then sent to the laboratory at the Chair of Plant Health, Estonian University of Life Sciences. Here, the wax and dead worker bees were separated. Following this separation, the samples were frozen again to maintain their integrity for future analyses.

4.2.3. Data collection and analysis (II)

The sampled apiaries were mapped, and subsequently, information requests were sent to the Agricultural Registers and Information Board (ARIB) to obtain data on the number of apiaries and hives within a 7 km radius, as well as the distance to every neighboring apiary. The data received from ARIB were then used to correlate the results of spore load of *P. larvae* with the density of surrounding apiaries.

4.3. Lab studies

4.3.1. Rearing of queen bees (IV)

The worker larvae were transferred from their cells to artificial plastic Nicot queen cups (grafting). After the grafting process, the frame with grafted cells (queen cups) was placed into a queen cell building colony, a very strong and young bee colony without a queen – with only nurse bees. This colony fed and took care of the developing larvae. Approximately on the fifth-sixth day after grafting, the grafted frames were taken out from the colony, each queen cell was caged for protection, and then transferred into the incubator. On the twelfth day after grafting, the queen bees emerged in the incubator.

4.3.2. Pesticide translocation in queen cells (III)

For the creation of queen cell cups in this study, wax sourced from a local organic beekeeping operation was utilized. The active compound, tebuconazole with a purity of 99.3%, was bought from SigmaAldrich. This chemical was dissolved in acetone and then integrated into the molten wax. The concentration of tebuconazole mixed in the wax was at 412 μg kg^{-1}, a level representative of field-realistic pesticide concentrations, based on an analysis of Estonian bee products (Raimets *et al.*, 2019). Following the incorporation of tebuconazole into the wax, queen cell cups were fashioned using a specific wooden dowel designed for this purpose. Different dowels were employed for shaping cups for the control group and the test group. The practice of forming queen cell cups from molten wax is a standard procedure in beekeeping. Subsequently, worker bee larvae, aged one to two day, were transferred into these freshly created cups. A frame containing both the control cups (n = 20) and the cups infused with tebuconazole (n = 20) was then placed into a queenright cell builder colony. The experiment was conducted in July, aligning with Estonia's primary honey flow season at the time of the study. The nearest conventionally farmed crops were located 5.4 kilometers away. The significant distance between the apiary and these conventional farming fields made it highly improbable that the bees would have ventured into the farmlands, thereby minimizing the likelihood of external tebuconazole exposure.

4.3.3. Effect of *Nosema* on queen development (IV)

The research was conducted in the summer of 2021, focusing on obtaining fresh spores of *Nosema apis* and *N. ceranae* from infected honey bee colonies. Worker bees from these colonies were collected for laboratory analysis. The first step involved extracting and crushing their midguts using a homogenizer, followed by filtering the resulting suspension through a 15 × 28.5 cm Universal Extraction Bag (Bioreba). A multiplex PCR (M-PCR) assay was then performed to confirm the species composition of the infection.

Once the species were confirmed, the spores were purified using a centrifugation protocol, achieving approximately 85% purity. Further refinement involved cleaning the suspension with a 10-micron filter to separate the final precipitate. A flow cytometer (BD Accuri C6) was used to quantify the spore count.

For the experimental setup, queen cell cups were made from organic beeswax sourced from a local producer to minimize the risk of pesticide contamination affecting the results. New queens of *Apis mellifera ligustica* were bred from one-day-old larvae, all originating from a single queen. The source colony was tested three times over three weeks to confirm it was free from *Nosema* infection. The colonies designated for nursing (royal jelly production and cell building) were also checked for infection over three weeks before the experiment to ensure they were *Nosema*-free. The one-day-old larvae were then grafted into the prepared cups, and the cell builder colony was monitored regularly until the worker bees sealed the cups.

Seven treatment groups were established, each consisting of ten larvae. The treatments were classified as "low" or "high" based on spore concentration: *N. apis* "low" (10,000 spores), *N. apis* "high" (50,000 spores); *N. ceranae* low, *N. ceranae* high; combined low and high treatments of *N. apis* & *N. ceranae*; and a control group. Once the cups were sealed, a 2 µL suspension containing the appropriate nosemosis agents (or ddH_2O for the control group) was injected through the wax walls into the royal jelly. The injection was carefully placed beneath the larvae, located using a strong light to visualize their position through the wax. Queen cell development was assessed on the tenth day post-grafting, and the cups were isolated using Nicot's queen cages before

being placed in an incubator (set to 35 °C and 65% humidity) until hatching.

4.4. Lab techniques

4.4.1. PCR (I, III)

A multiplex PCR (M-PCR) assay was conducted to identify *Nosema* species, using a 2 µL DNA sample. For detecting *N. ceranae*, the primer MnCeranae-F with the sequence 5′-CGTTAAAGTGTAGATAAGATGTT-3′ is used, resulting in a fragment size of 143 base pairs (bp). To identify *N. apis*, the primer MnApis-F with the sequence 5′-GCATGTCTTTGACGTACTATG-3′ is utilized, yielding a fragment size of 224 bp. The universal reverse primer, Muniv-R, has the sequence 5′-GACTTAGTAGCCGTCTCTC-3′, and it is used in conjunction with both forward primers without specifying its fragment size (Fries *et al.*, 2013).

The PCR cycle began with a 2-minute initial denaturation at 95 °C. This was followed by 35 cycles, each consisting of: 30-second denaturation at 95 °C, 30-second annealing at 57 °C, 1-minute elongation at 72 °C.

The cycle concluded with a final elongation step of 5 minutes at 72 °C. The resulting M-PCR products were then visualized using a 2% agarose gel.

4.4.2. qPCR (II)

For qPCR quantification standards, *P. larvae* ATCC 9545 was used (Rossi *et al.*, 2018). This strain was cultured on Sheep Blood Agar (Biolife Italiana, Milan, Italy) and incubated at 37 °C for 2–5 days in the presence of 9% CO_2. Single colonies were sub-cultured on the same medium under identical conditions. Biomass from the second culture was suspended in 10 ml of sterile phosphate-buffered saline (PBS, pH 7.4) and heated at 80 °C for 10 minutes to kill vegetative cells. The spores were then harvested by centrifugation at 12,000 rpm for 5 minutes, followed by two washes with sterile PBS. Spore counts were determined via plate count on Sheep Blood Agar and serially diluted to inoculate

autoclaved hive debris (121 °C for 15 min), which was then used for qPCR quantification standards.

A modified version of the method by Rossi *et al.* (2018) was employed to detect *P. larvae* in hive debris. The modification involved replacing proteinase K lysis with bead-beating for faster and more cost-effective lysis. In brief, 100 mg aliquots of hive debris were transferred to 2 ml Eppendorf tubes containing 200 µl of T1 buffer (Macherey–Nagel GmbH & Co. KG, Düren, Germany) and 100 mg of sterile 200 µm glass beads (Merck). This mixture was homogenized using a Tissue Lyser II (Qiagen, Hilden, Germany) at 30 Hz for 3 minutes. Bead-beating was chosen for its shorter lysis time (3 minutes versus 1 hour for proteinase K) and lower cost, with comparable DNA extraction results confirmed by statistical analysis of Ct values from samples artificially contaminated with *P. larvae* spores. Following homogenization, 200 µl of B3 buffer (Macherey–Nagel) was added to the mixture, which was then centrifuged at 12,000 rpm for 10 minutes. The supernatant was transferred to a clean Eppendorf tube containing 210 µl of pure ethanol, mixed, and left to precipitate for 10 minutes. The mixture was passed through a DNA extraction column according to the instructions in the NucleoSpin Tissue Kit (Macherey–Nagel). The same method was used to prepare qPCR standards from hive debris samples artificially inoculated with *P. larvae* spores.

The quantification standards were generated by extracting DNA from triplicate series of hive debris inoculated with *P. larvae* spore dilutions ranging from 10 to 1,000,000 CFU/g (Rossi *et al.*, 2018). Amplification and calibration curves were created as described by Rossi *et al.* (2018), allowing for correction of variability in DNA extraction yields. Calibration curves were included in each qPCR run to quantify *P. larvae* in the samples using the QuantStudio 5 software, a standard practice for absolute quantification in qPCR (Rossi *et al.*, 2018).

qPCR was performed using a QuantStudio 5 thermal cycler (Applied Biosystems, Thermo Fisher Scientific, Rodano, MI, Italy) in 20 µL reactions, following the method outlined by Rossi *et al.* (2018). The qPCRBIO SyGreen Mix Separate–ROX qPCR Master Mix (PCR Biosystems, London, UK) was used, along with primers PLAup (5'-TTCGGGAGACGCCAGGTTA-3') and PLAdw (5'-CTTTCATGACTTCTTCATGCGAAG-3') at final concentrations

of 0.25 µM and 0.15 µM, respectively. Calibration curves for *P. larvae* quantification were established using DNA extracted from autoclaved hive debris spiked with *P. larvae* ATCC 9545 spores at concentrations ranging from 1 to 1,000,000 CFU/g. Quantification results for *P. larvae* spores in each sample were provided by the QuantStudio 5 Design and Analysis software v1.5.2. Non-specific amplification was ruled out via melting curve analysis, which produced a specific melting peak for *P. larvae* at 84 ± 1.5 °C, verified by testing *Paenibacillus* species with the highest sequence similarity in the target region (Rossi *et al.*, 2018).

4.4.3. Flow-cytometry (I, IV)

A BD Accuri C6 flow cytometer was used to count the number of spores in honey bees. Flow cytometry distinguishes between spore species primarily based on their size (Kullman & Küngas, 2018). Although there is some overlap in spore size between species, the cytometer plot generates distinct curves based on the size distribution of spore-like particles, allowing identification based on the majority of the base area (Figure 3). In cases of mixed infections, the total spore count was reported.

To prepare the samples, a 50 µL aliquot was taken from the DNA isolation suspension, diluted with 1 mL of distilled water, and 50 µL was used for spore counting. A disposable 10 µm sieve was used to filter out debris before spore counting. To calculate the number of spores per bee, we created the following formula:

$$\boldsymbol{x} = \frac{\mathbf{3000 \times 1050}}{\mathbf{20 \times 50 \times 50}} \times \text{SA (spores in the sample).}$$

The formula is explained as follows: 3000 (3000 µl of water added to facilitate homogenisation), 1050 (final mixture from which 50 µl of spore suspension was removed was diluted with 1000 µl of water), 20 (number of bee abdomens used), 50 (50 µl of spore suspension used in the dilution), and 50 (50 µl of the filtered spore suspension was loaded into the flow cytometer for spore counting). The accuracy of the results was confirmed by comparing flow cytometry counts with microscopic spore counts from five samples of each infected species.

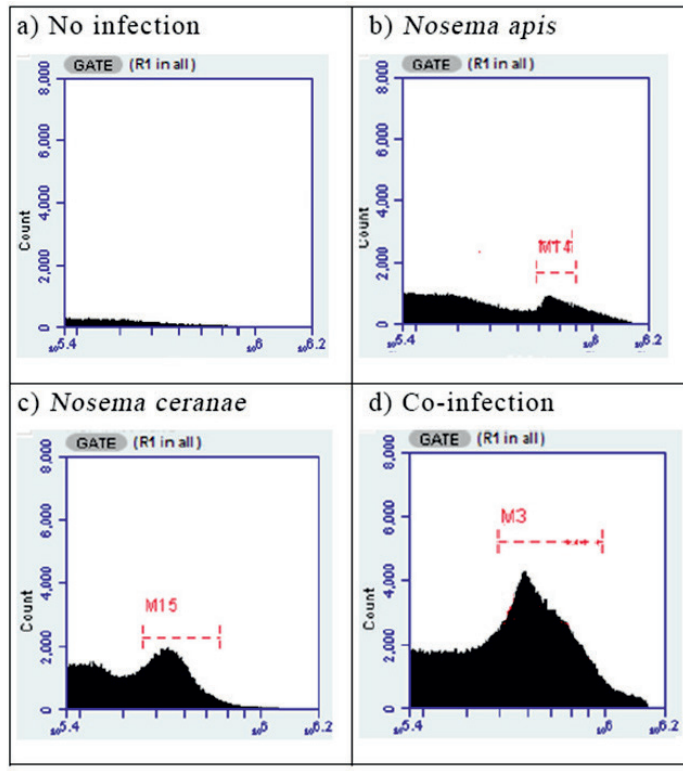

Figure 2. Graph of the flow cytometer result according to the number of *Nosema* sp. spores of different sizes: a) No infection, b) *N. apis* range (M14), c) *N. ceranae* range (M15), d) Co-infection range (M3).

4.5. Pesticide analyses (III)

Pesticides residues in this study was analized at the Institute of Food Safety, Animal Health and Environment "BIOR". The UHPLC-MS/MS assay was performed using an Ultimate 3000 high performance liquid chromatograph (Thermo Scientific, MA, USA) coupled to TSQ Quantiva tandem mass spectrometer (Thermo Scientific, MA, USA).

4.6. Statistical analyses

The Kruskal-Wallis test was used to assess the statistical differences in spore number per single honey bee in Estonia and Latvia and confirm the accuracy of the flow cytometry results (**I**) also to compare single hive samples with pooled samples (**II**).

The chi-square test, was used to examine the statistical significance of differences between *N. ceranae* and *N. apis* distribution changes in Latvia (**I**).

The chi-square test was used to compare the distribution of pooled and single hive samples in different infection categories (negative, detected, <LOQ, low, high, very high) (**II**).

Spearman's rank-order correlation was used to assess whether the number of hives in the apiary, the number of neighboring beehives, and their distances were correlated with the number of *P. larvae* spores in the samples (**II**).

For calculating the hazard quotient (HQ), the wax toxicity calculation tool (Bee Tox Wax) was used (Toolbox, 2020) (**III**). The aim of HQ was to evaluate the potential exposure to a pesticide and to set a level at which no adverse effect is expected. When using the "Bee Tox Wax" tool, the median lethal dose (LD_{50}) is a quantitative indicator of the pesticide toxicity. In this case, HQ values under 250 were shown to be slightly toxic to bees and the wax can be re-used.

5. RESULTS

5.1. Distribution of diseases

5.1.1. *Nosema* sp. (I)

Among the 30 apiaries sampled in Estonia (2017), the prevalence of *N. apis* was 43%, *N. ceranae* was found in 17%, and 7% of the apiaries had co-infections of both species. In 33% of the Estonian apiaries, no *Nosema* species were detected.

In Estonia, the median spore count for honey bees with co-infections varied from 0-12 million (Figure 2, **I**). For single infections, counts were about 6 million for *N. apis* and 4 million for *N. ceranae*. Statistical analysis revealed no significant differences in spore counts between the two *Nosema* species or between single and mixed infections.

Conversely, in Latvia, with a sample size of 60 apiaries (2018), the distribution was different: 15% of the apiaries tested positive for *N. apis,* 47% for *N. ceranae*, and 18% had co-infections. In the remaining 20% of the Latvian apiaries, neither of *Nosema* species were detected during the sampling period.

A significant change in the distribution of *Nosema* species in Latvia was observed ($\chi^2 = 35.71$, $p < 0.001$) between 2013 and 2018. In 2013, 65% of collected samples were co-infected (unpublished data from the Latvian Beekeepers Association), with 15% of the samples infected with *N. apis* and 20% infected with *N. ceranae*. In contrast, by 2018, the prevalence of *N. ceranae* infections in Latvian apiaries had increased considerably (Figure 3, **I**). In Latvia, the median spore count in co-infected honey bees was around 9 million. For single infections, *N. apis* had a median count of approximately 1.7 million spores per bee, and *N. ceranae* had about 2.4 million, both significantly lower than co-infection counts.

5.1.2. *Paenibacillus larvae* (II)

In total, 179 samples were collected from 13 out of 15 counties in Estonia. Two counties did not participate due to no local county

beekeeping inspectors there. The results revealed that 52.5% of the analyzed samples tested positive for *P. larvae*. Most of these samples exhibited a low infection rate, with spore counts below 10 CFU/g, corresponding to cycle threshold (Ct) values higher than the standards set for 10 *P. larvae* spores. The calibration curve for all PCR runs displayed linearity over a range of at least five orders of magnitude, from 100 CFU/g to 1,000,000 CFU/g spores. However, spore counts below 100 CFU/g and above 1,000,000 CFU/g were considered unreliable due to the limitations in the linear range of the calibration curve. The limit of detection (LOD) for the PCR assay and the calibration curves for the primers had previously been determined by Rossi *et al*. (2018) as 10 CFU/g spores of *P. larvae* in hive debris. As a result, samples with Ct values corresponding to 10 CFU/g spores or higher were categorized as "detected." Based on the spore counts from hive debris, spore loads were classified as follows: negative, detected <10 CFU/g, <LOQ (100 CFU/g), low (152–204 CFU/g), high (623–932 CFU/g), and very high (183,000 to >1,000,000 CFU/g). Intermediate spore counts between 1,000 and 182,000 CFU/g were not identified in the samples and were therefore excluded.

Out of all the tested samples, 48.2% were negative, 27.1% were positive but below the limit of quantification, 9.4% fell below the classification threshold, and 5.9% contained varying spore loads ranging from "low" to "very high." As illustrated in Figure 1 (**II**), the proportions of negative samples and those with detectable spore levels varied significantly (χ^2 = 31.5, $p < 0.001$) between pooled and single hive samples. Figure 2 (**II**) further shows that pooled hive samples had significantly lower median spore counts compared to individual hive samples (KW-H (1;93) = 9.40; $p = 0.002$), suggesting that pooled samples might disperse spores from highly contaminated hives.

There was no statistically significant correlation ($r = 0.32$; $p = 0.29$; $r^2 = 0.10$) between the number of registered beehives per county and the proportion of *P. larvae*-positive samples (Figure 3 (**II**)). The spore load was positively correlated with the number of neighboring apiaries and the number of beehives within a 7 km radius of the sampled apiary, and negatively correlated with the apiary's longitudinal location (Table 1 (**II**)). However, there was no correlation between spore loads in hive debris samples and factors such as the number of beehives in the target

apiary, the distance to the closest neighboring apiary, or the average distance to neighboring apiaries.

Despite the presence of very high spore loads in some hive debris samples in spring, no clinical signs of AFB were observed in the colonies during the following summer (Table 2 (**II**)). Among the 42 individually sampled bee colonies, including those with high *P. larvae* spore loads, no AFB outbreaks were reported by beekeepers over the summer. Still, one colony with an exceptionally high spore load did experience issues: the colony remained small throughout the summer and eventually suffered queen failure.

5.2. Factors affecting queen breeding

5.2.1. Pesticides in wax (III)

Tebuconazole residues were detected in the entire queen cell wax (0.19 mg kg^{-1}) (Table 2, **III**), indicating successful assimilation into the wax at the beginning of the experiment. Despite the detectable tebuconazole in the queen cell cups, no residues were found in the honey bee queen larvae or the newly emerged queens. However, lower concentrations of tebuconazole were identified in royal jelly samples, with a measured concentration of 0.08 mg kg^{-1} (0.08 μg per bee), which is 2.4 times less than that in the wax.

The Hazard Quotient (HQ) was calculated to assess the potential toxicity of tebuconazole to honey bee queens, based on its concentration in the wax. With an initial wax concentration yielding an HQ value of 1, the wax contamination level is considered low. The detected concentration of 0.19 mg kg^{-1} (0.19 μg per bee) in the queen cell wax is unlikely to cause direct mortality but may adversely affect queen homeostasis at this sub-lethal concentration. The acute lethal contact dose of tebuconazole over 48 hours is known to be 200 μg per bee (Toolbox, 2020). Given that the HQ value in this case was 1, the wax is deemed slightly contaminated but still suitable for use and recycling in beekeeping. According to the Bee Tox Wax tool, any HQ value below 250 signifies low pollution levels (Toolbox, 2020), indicating that the wax can be safely used and recycled in beekeeping operations.

5.2.2. *Nosema* sp. (IV)

The hatching rate of honey bee queens after *Nosema* treatment varied. High spore load treatments with *N. apis* resulted in a 0% hatching rate, whereas the same spore load of *N. ceranae* maintained an 80% hatching rate. Co-treatment with high spore loads led to a 10% hatching rate. Lower spore load treatments for *N. apis, N. ceranae*, and their combination resulted in higher hatching rates of 60%, 70%, and 70%, respectively (Figure 1, **IV**). Queens exposed to high doses of *N. apis* and *N. ceranae* spores that did not hatch showed developmental abnormalities at various stages, preventing their maturation (Figure 2, **IV**). The study also investigated whether pathogen spores could undergo metamorphosis and be detectable in hatched adult queens, but no spores were detected in the hatched queens.

6. DISCUSSION

6.1. Persistence, distribution and change of *Nosema* species share over time

We showed the persistence of *Nosema* infections over time. The finding that only 20% of the samples no longer contained microsporidian spores, highlights the stubborn nature of the infection (Holt & Grozinger, 2016; Mazur & Gajda, 2022). Once a bee colony is infected, the pathogen remains persistent, causing ongoing moderate to acute stress to the colony. Since nosemosis is a spore-forming disease, almost any organism in contact with the spores can act as a vector. Moreover, the spores thrive in various environments, including water (MacInnis *et al.*, 2021), honey, and beeswax, though their viability may differ depending on the medium (MacInnis *et al.*, 2020). Beekeepers themselves can also unintentionally spread the spores if they fail to properly sterilize tools, clothing, and footwear – especially, as many professional beekeepers manage numerous apiaries. Ideally, after working with each apiary, tools and clothing should be sterilized to reduce the risk of pathogen transmission. Like other pathogens and parasites, *Nosema* can also spread through honey bee behavior, such as late-autumn robbing when bees enter other hives. There is also evidence that the pathogen can spread through drone sperm (Yániz *et al.*, 2020).

Contrary to the prevailing trend of *N. ceranae* dominance over *N. apis* in many parts of the world (Martín-Hernández *et al.*, 2018; Pasca *et al.*, 2019; Paxton *et al.*, 2007), the present findings for Estonia (2017), reveal a persistent prevalence of *N. apis*. This situation contrasts with the situation in Latvia (2018), where *N. ceranae* was found to be more prevalent, indicating a geographical variation in the dominance of *Nosema* species although, these studied countries are two small neighboring countries. In Latvia, DNA-based identification from 2013 to 2018 clearly indicates that *Nosema ceranae* was able to outcompete other competitive species within five years. Although the Estonian results challenge the narrative of a complete takeover by *N. ceranae*, our new dataset, collected by Naudi *et al.* (unpublished), suggests that the takeover process by *N. ceranae* was also ongoing in Estonia.

Historical data, such as the gradual northward movement of *N. ceranae* since its discovery in Spain in 2005 (Klee *et al.*, 2007), provide valuable context for understanding current distributions. However, methodological differences, such as the reliance on light microscopy in earlier studies, which has a lower accuracy compared to molecular detection techniques, must be considered when interpreting these trends.

The significant numbers of positive samples for *N. ceranae* in Serbia from 1992 to 2017 (Matović *et al.*, 2020) and its dominance in Canada (Emsen *et al.*, 2016) further illustrate the widespread impact of this pathogen, suggesting that *N. ceranae* was present in Europe historically before 2005. These findings indicate that *N. ceranae* has had a steady and resilient presence for decades, thriving in both colder and warmer climates. Since some regions in Canada have climate similar to Estonia and Latvia due to their similar latitudes, it seems likely that *N. ceranae* will establish a permanent population in our climate - if it hasn't already occurred yet. Examining nearby countries with similar cold climates, such as Finland, reveals that *N. ceranae* quickly became dominant after its initial detection in 1998. This highlights the species' ability to establish itself rapidly in new environments (Paxton *et al.*, 2007).

Our methodology entailed collecting samples in May, during the transitional period from old winter bees to new young nurse bees. It's known that winter bees have a larger fat body (vitellogenin) (Aurori *et al.*, 2014) to survive harsh periods. The interpretation of the results must consider the timing of sample collection – due to differences in high peak of spore loads of these two species, it might have affected our results. The *Nosema* spore loads depend not only the virulence of the pathogen (Gisder *et al.*, 2017), but also the physiological changes that honey bees, as hosts, undergo across seasons (Forsgren and Fries, 2010). Seasonality is a highly debated topic in the development of nosemosis in honey bee hives. It is widely accepted that *N. apis* exhibits strong seasonality, with the highest spore loads and pathogen development occurring in spring and autumn, coinciding with periods of increased colony activity and environmental stress (Ii & Quandt, 2020). While *N. ceranae* is considered more of a stressor throughout the entire season, including winter (Emsen *et al.*, 2020). This raises the question of whether seasonality may have influenced the balance between these Nosema species in our study.

The spore counting experiment with flow cytometry showed that the number of spores was highest during co-infection. The arise of spore counts may be caused by the more aggressive proliferation of either or both of the pathogens in competitive conditions (Forsgren & Fries, 2010; Milbrath *et al*., 2015). Moreover, co-infections can alter the host organism's behavior and physiology in a way that facilitates the spread and proliferation of both pathogens (Ii & Quandt, 2020). For example, sick bees may collect more pollen resources, providing more resources for the reproduction of *Nosema* spores – since there are more vectors that can cause subsequent infection (Botías *et al*., 2013; Pasquale *et al*., 2016).

As of today, no efficacious method has been successfully formulated to eradicate *Nosema* sp. from hives. Historically, antibiotics like fumagillin were considered a potential solution (Huang *et al*., 2013), but their use has been banned for about a decade. A recent study (Prouty *et al*., 2023) reaffirms that antibiotics are not a viable option for controlling *Nosema* infections. Similarly, other recent research has shown that fumagillin is not only ineffective against *N. ceranae* but also poses significant toxicity risks to bees and is no longer recommended for use in many countries (Peirson & Pernal, 2024).

To effectively mitigate nosemosis in honey bee colonies, the most reliable approach currently involves maintaining strict biosecurity protocols and ensuring optimal sterility during colony management (Formato *et al*., 2022). Moreover, if the *Nosema* infection is proven, it would be advisable to carry out a specific shook-swarm method of the bee colony in early spring. Additionally, ensuring a high influx of proteins for the bees, for example, in the form of pollen, as confirmed by Tritschler *et al*., (2017).

A comprehensive review on microbial threats to honey bee health emphasizes the importance of integrated pest management strategies, highlighting the multifaceted challenges posed by pathogens and the necessity for holistic approaches to bee health management (He *et al*., 2021). New technologies, such as RNA interference (RNAi) based on a specific gene-silencing approach, are under development (Burnham, 2019). Further advancements in RNAi technology include the use of engineered symbionts to deliver RNAi targeting *Nosema* (Huang *et al.,* 2023). Another innovative therapeutic approach is the transferrin-

mediated iron sequestration strategy, which leverages the iron homeostasis mechanisms in honey bees (Rodríguez-García *et al.*, 2021)

6.2. The role of hive debris and environmental factors in detecting and managing *Paenibacillus larvae*

Spring debris analyses indicated widespread but relatively low-level contamination of *Paenibacillus larvae* spores in Estonian beehives. The predominance of samples with low spore counts (detected or below the limit of quantification) suggests a high incidence of sub-clinical infections, which are challenging the detection and management but crucial for understanding disease dynamics. Earlier simple PCR based methods resulted only in understanding of the presence or absence of the pathogen giving no clues about the spore load (de Graaf *et al.*, 2006). The development of DNA-based quantitative analysis techniques, as demonstrated by Rossi *et al.* (2018), has advanced analyses to a new level, highlighting the utility of these techniques for early detection of infections and proactive management.

The outbreak of AFB in honey bee colonies can depend on several factors, with the hygienic behavior of the colony playing a crucial role. It is hypothesized that today's honey bees are stronger than those of previous decades, partly due to selective breeding for improved traits like hygienic behavior (Harpur *et al.*, 2019; Maucourt *et al.*, 2021; Spivak & Gilliam, 2015). This enhanced hygienic behavior enables bees to recognize and address various problems within the colony, which may reduce the spread of diseases and lower the likelihood of outbreaks. By detecting and removing diseased brood before stressors can spread, these stronger bees increase the colony's overall resistance to diseases.

Changes in the virulence of *P. larvae* could also influence disease expression. Studies have shown that different genotypes of *P. larvae* exhibit varying levels of virulence. For example, the genotypes ERIC I and ERIC II differ significantly in pathogenicity, with ERIC II being more virulent and killing host colonies faster than ERIC I (Djukic *et al.*, 2014). Additionally, the genotypes ERIC III and ERIC IV, though less prevalent, have also been identified, adding to the genetic diversity of *P. larvae* (Genersch *et al.*, 2006).

Our study supports this complexity; despite observing very high spore loads in some samples, none of the beekeepers reported the evident clinical signs of AFB in the colonies during the subsequent summer. Interestingly, these tested colonies were not specifically selected for hygienic behavior. It's worth considering that, although typically Estonian beekeepers may not specifically select for hygienic behavior, the queens are often replaced, and queen breeders still choose their lines (personal communication with beekeepers and local queen breeders), which may have led to an improvement in colony health even without intentional selection. Factors such as colony strength, genetic resistance, and environmental conditions may influence the transition from latent infection to overt the disease (de Graaf *et al.*, 2013).

When considering sampling methodologies for AFB surveillance in honey bee colonies, it is crucial to distinguish between the interests of individual beekeepers and national or regional monitoring programs. For beekeepers who need to verify a suspicion of infection, modern self-use tests (The Vita American Foulbrood Diagnostic Test Kit), which complement traditional indicators like sticky, foul-smelling brood and purulent hive conditions, are effective tools. These tests provide rapid and accessible means to confirm the presence of the pathogen. For routine monitoring at national level, commercially available rapid tests could be well suited. For example, swab testing has proven effective in identifying *P. larvae* (Mackay *et al.*, 2023). If beekeepers are not willing or able to purchase these tests themselves due to their relatively high cost, there is the option to obtain them through a national program. Routine monitorings however could be facilitated by further development of affordable, reliable, and non-invasive techniques for widespread adoption. Various studies have demonstrated the analytical capabilities of different matrices such as honey, wax, and hive debris (Forsgren & Laugen, 2014; Gajger & Bakarić, *et al.*, 2024). Testing methods must be suitable for broad application to ensure they are practical for regular use by beekeepers.

One key consideration in sampling methodology is whether to sample each hive individually, use random hive selection, or employ pooled sampling. Our results indicate that pooled samples may dilute the spore concentration from highly infected hives, which could lead to an underestimation of infection levels. Therefore, individual hive sampling provides a more accurate reflection of infection dynamics within colonies.

Although it is known that *P. larvae* can spread more easily in regions with higher honey bee densities (Stephan *et al.*, 2020; Zabrodski *et al.* 2022), our study did not show any explanatory power for the pathogen's spread. However, there was a positive correlation between spore load and the number of neighboring apiaries, as well as the total number of beehives within a 7 km radius. This may be due to honey bee population densities may facilitate the spread of *P. larvae*, possibly due to increased opportunities for robbing and drifting between colonies (Lindström *et al.*, 2008). There are indications that areas with a high number of beekeepers employing diverse management practices can see varied impacts on disease spread (Kahane *et al.*, 2022). This variability in management practices like hygienically selected queens and regular monitoring can affect, how effectively colonies can resist or succumb to infections.

Environmental factors also contribute to AFB spread. Weather conditions, availability of forage, and overall colony health can influence the susceptibility of bees to infection. For example, poor nutrition due to limited forage can weaken bee immune systems, making them more prone to diseases (El-Seedi *et al.*, 2022; Ricigliano *et al.*, 2022).

Our research also revealed that the viability of *P. larvae* is significantly affected by meteorological conditions. Despite Estonia's small size, it spans distinct climatic zones, classified by Tóth *et al.* (2016) into two primary areas: boreal to subboreal and northern subcontinental. The north-south directional climate variation is subtle, yet there's a pronounced transition from a more severe continental climate in the east to a milder maritime climate in the west. Our study observed a notable rise in spore counts in the western maritime climate, suggesting that its milder weather may foster the spread of American foulbrood (AFB), in contrast to the harsher conditions found inland. Fries (2010) demonstrated that the susceptibility of bees to certain pathogens can vary with climate, potentially due to differences in food availability or the bees' ability to adapt to humid versus dry conditions. Rowland *et al.* (2021) demonstrated that *Varroa* mite infestations increased with higher temperatures, but decreased with heavy rainfall and wind. At the same time contrastingly, the risk of AFB, was not significantly affected by any weather variables and lacked any relationship with time (Rowland *et al.*, 2021). Considering climate change, warming temperatures may increase the prevalence of diseases and reduce the effectiveness of

treatments. Higher temperatures can promote the growth and spread of pathogens, while also altering bee behavior and physiology, potentially making colonies more vulnerable to infections as noted by Jongh *et al*., (2022). Le Conte & Navajas (2008) noted that shifts in temperature and precipitation patterns can alter the prevalence and virulence of pathogens like *Nosema* sp. and *Varroa* mites. Specifically, warmer temperatures can increase the reproduction rates and spread of pathogens like *Nosema* and *Varroa* mites, while varying conditions of humidity and precipitation can affect food availability and the honey bees` ability to cope with these stressors (McAfee *et al*., 2024; Smoliński *et al*., 2021).

Developing non-destructive predictive methods is crucial for enabling timely preparation and raising beekeepers' awareness. Early readiness allows beekeepers to take appropriate measures to protect their colonies and mitigate the impact of diseases. Non-invasive techniques, such as hive debris sampling, can enable beekeepers to monitor colony health without resorting to invasive procedures that may further stress the bees. Techniques such as environmental DNA (eDNA) analysis (Ribani *et al*. 2020), which uses DNA from honey to detect pathogens, offer innovative solutions for early disease detection. They demonstrated that eDNA in honey can be used to identify and monitor honey bee pathogens and parasites, which helps in establishing early and effective containment measures.

6.3. Tebuconazole contamination in wax

The persistence of synthetic pesticides, particularly in lipophilic matrices like beeswax, poses significant risks to honey bee health. Beeswax, due to its non-polar, fat-soluble nature, can store agrochemicals, allowing pesticides to accumulate and potentially threaten honey bee colonies (Milone & Tarpy, 2021; Mao *et al*., 2013; Mullin *et al*., 2010). Although pesticide translocation between materials is well-documented, our study shows that this risk may not be as significant as previously thought.

Our research demonstrated that tebuconazole, when introduced solely through treated wax, did transfer to royal jelly, but there was no further transmission to queen larvae or newly emerged queens. The bees' construction of the combs likely reduced the concentration of tebuconazole that the larvae were exposed to. Despite an initially high concentration in the wax, the levels detected in the overall queen cell

wax were notably lower, indicating a dilution effect from the addition of new wax.

Various chemicals have been shown to migrate from wax into royal jelly (Fuente-Ballesteros *et al.*, 2023; Milone & Tarpy, 2021). Our study demonstrated this migration, but with a notable decrease in concentration as it translocates from wax to royal jelly. This suggests a potential transfer pathway, though at levels that may pose minimal risk to honey bee queens' survival. In addition to migration through wax, royal jelly contamination may also occur via nurse bees. However, research has shown that even when nurse bees are fed contaminated pollen and nectar, they can still produce uncontaminated royal jelly for queen bees (Böhme *et al.*, 2018).

Interestingly, although tebuconazole was present in the wax and to smaller extent in royal jelly, no residues were detected in the queen larvae or newly emerged queens. This points to a possible metabolic mechanism within the bees that can eliminate tebuconazole, which warrants further investigation (Johnson *et al.*, 2013; Mullin *et al.*, 2010). The sex and caste of the subject should also be considered, as these factors may differ significantly between worker bees and queens (Jürison *et al.*, 2022). The biological and chemical processes governing the transfer of substances between materials may naturally limit the amount of tebuconazole reaching the queen larvae (Fuente-Ballesteros *et al.*, 2023). Since royal jelly is produced by worker bees from glandular secretions, its production process could reduce tebuconazole concentration, potentially due to the bees' metabolic activities or selective absorption from their environment. Moreover, the unique biochemical composition of royal jelly, rich in proteins, fats, and vitamins, may shield the larvae from tebuconazole exposure (Botezan *et al.*, 2023; Collazo *et al.*, 2021). Certain components within royal jelly could bind to or neutralize tebuconazole, thus decreasing its bioavailability to larvae.

A key factor in our study was the methodology for analyzing the movement of tebuconazole between materials. When reviewing the initial mixture, it became apparent that bees diluted the treated wax with new wax, reducing contamination levels. Several studies have reported on wax contamination and its adsorption properties within the hive environment (Fuente-Ballesteros *et al.*, 2023; Liu *et al.*, 2023; Traynor *et al.*, 2016). The production of royal jelly and the bees' metabolic mechanisms appear

to serve as natural filters, preserving the purity of royal jelly even when environmental conditions are compromised (Baptista *et al.*, 2023). This phenomenon can be further understood by considering the lipophilic nature of certain contaminants, like tebuconazole, which tends to accumulate in non-polar media. In our study, the highest concentrations of tebuconazole were found in beeswax (a highly non-polar matrix), while lower levels were detected in royal jelly, which contains up to 6% fats, and the lowest levels were found in larvae, whose composition is primarily water and protein. This suggests that the chemical properties of tebuconazole cause it to accumulate more in lipid-rich environments, thereby minimizing its presence in royal jelly and larvae.

The Hazard Quotient (HQ) was used to assess the potential toxicity of tebuconazole found in the wax. The concentration detected in queen cells was 0.19 mg/kg (0.19 μg per bee), indicating that while it should not cause direct mortality, this sub-lethal concentration could affect queen homeostasis. Given that 200 μg per bee is considered the acute lethal dose after 48 hours, the HQ in our study was 1, suggesting that the wax was mildly contaminated but still within acceptable limits for use in beekeeping and recycling. According to the Bee Tox Wax tool, any HQ value below 250 (toolBox 2020) is deemed low-polluted, meaning the wax is still suitable for use and recycling in beekeeping operations. Although recycling contaminated wax in beekeeping may be permissible under certain HQ thresholds, it raises concerns about the long-term sustainability and health of bee colonies. Continuous exposure to low levels of contaminants can lead to residue accumulation in hive products and within the bees (Raimets *et al.,* 2020), potentially affecting honey quality (Karise *et al.*, 2017) and the colony's resilience (Bryden *et al.*, 2013).

While acute toxicity thresholds provide important safety markers, they do not capture the full scope of chronic and sub-lethal effects that pesticides may have on bee health. Even at low concentrations, tebuconazole may disrupt the hormonal balance of queen bees, impair their reproductive abilities, and weaken their immune systems (Zubrod *et al.*, 2010). These effects could cascade through the colony, leading to reduced brood viability and overall impaired colony performance (Stoner & Eitzer, 2013). Moreover, the presence of multiple contaminants, as is common in real-world conditions, can lead to synergistic effects that exacerbate overall toxicity (Doublet *et al.*, 2015; Siviter *et al.*, 2021). Bees are often

exposed to a combination of agrochemicals, and the interactions between these substances can amplify their harmful effects (David *et al.*, 2016). For instance, studies have shown that when tebuconazole is combined with the neonicotinoid thiamethoxam, the mixture's toxicity increases synergistically as reviewed by Schuhmann *et al.* (2022).

6.4. Impact of *Nosema* on queen bee development and colony health

An important factor in queen rearing is the transmission of diseases to the offspring. *Nosema* can reside in the hypopharyngeal glands of nurse bees and thus be transmitted through the royal jelly (Higes *et al.*, 2008; Marín-García *et al.*, 2022). Traver & Fell (2012) have demonstrated that *Nosema* DNA can be found both in the food of queen bees and later in emerged queen bees – which has left open the hypothesis whether the *Nosema* spores are capable of moving from the royal jelly given to the developing queen larvae and further into the adult queens. We demonstrated that infecting royal jelly with *Nosema* sp. seriously affects the queen breeding success, the emerging rates were affected. Moreover, this effect is both species- and dose-specific.

The species-specific effects of *Nosema* sp. on honey bee health has also seen by Gisder *et al.* (2017) and Paxton (2010). Our complete failure of queen hatching under high *N. apis* spore load treatment highlights the severe impact this pathogen can have on queen development. However, it should be mentioned, that commonly, the queen rearing process is not carried through during the times of *N. apis* naturally high peaks in early spring (Botías *et al.*, 2013; Martín-Hernández *et al.*, 2012) and *N. apis* high concentration infection of queen larvae is unlikely. During swarming, which is when queen rearing usually takes place, *N. apis* levels in hives are generally low (Botías *et al.*, 2013; Martín-Hernández *et al.*, 2012).

Similar works have not been done before, however, there are several studies that demonstrate the danger of *Nosema* to the honey bee queens. For example, Hassanein (1951) demonstrated the negative impact of *N. apis* on queen bees a long time ago, noting that spore producers were observed in the mid-gut of queen bees as early as 12 hours after infection. Since *N. ceranae* is a more acute source of infection (Alaux *et al.*, 2011), there have been a few recent studies highlighting its detrimental effects

on queen physiology. However, experiments involving queen bees require a high level of professionalism in both beekeeping and scientific research, and they are costly and highly seasonal (Büchler *et al*., 2023), which likely explains the limited number of studies on this subject.

The observed low hatching rate under treatment with both *Nosema* species, even at lower spore loads, indicates that the presence of both pathogens significantly impacts the bees' ability to cope with infections at the colony level. Botías *et al*. (2013) found that the combined presence of these pathogens exacerbates infection severity, while Broccard-Bell *et al*. (2020) reported similar findings, demonstrating an additive effect of co-infection that stresses bee colonies further.

This phenomenon highlights the complex interactions between pathogens within bee hosts and the potential for compounded negative effects on bee health, a topic that has received increased attention in recent studies exploring multi-pathogen dynamics in bee populations. Siviter *et al*. (2021) found that multi-pathogen infections can significantly exacerbate the negative health effects on bee populations. They demonstrated that co-infections lead to increased mortality rates and reduced overall colony health.

We did not detect *Nosema* sp. DNA in the emerged queens through Multiplex-DNA analysis. It can be hypothesized that if the royal jelly of the queen bee is contaminated at an early stage of larval development, the larva undergoes a pre-pupation phase before pupation. During this phase, the larva may excrete waste to the bottom of the cell, potentially reducing the likelihood of pathogen retention within the developing queen (Chen *et al*., 2023), and then metamorphosis begins. Based on our observations, a certain amount of royal jelly remains at the bottom of the cell. Unfortunately, in this study, we were unable to extend the experiment to determine whether this leftover royal jelly could serve as a source of reinfection for the newly emerged queens.

Determining the precise number of spores required to induce acute stress and disease outbreak in honey bees remains a relatively grey area, and most of studies are conducted with worker brood and adults. Aufauvre *et al*. (2014) found that sublethal doses of *N. ceranae* can significantly impair honey bee health by reducing lifespan and increasing susceptibility to other pathogens. Pent *et al*. (2023) focused on the spread

and development of *Nosema* spores within bee colonies, demonstrating that high spore loads can lead to significant colony stress and increased pathogen transmission. The 10,000 and 40,000 spores inoculated by us into the queen bee's royal jelly was similar to those inoculated by Eiri *et al*. (2015). In their research, Eiri *et al*. tested whether *N. ceranae* could infect honey bee larvae and examined the subsequent effects on adult longevity. They found that larvae exposed to the spore loads of 10,000 and 40,000, developed spores in their midgut cells, with 55% and 60% infection rates, respectively. The infection significantly reduced the longevity of the adult bees that emerged from these larvae.

CONCLUSIONS

Honey bee health and the sustainability of beekeeping depend on many complex factors. Beyond traditional epidemiological research questions, the early detection of pathogens - before the outbreak of relevant diseases - can significantly aid in the management of healthy beehives. Innovative analytical methods not only enhance the accuracy of analysis results but also raise new questions about the tolerable number of latent spores in hives, factors influencing the spread of pathogens, and ongoing debates regarding preventive tools available to beekeepers, even when they are aware of potential threats to their honey bee colonies.

An aspect that has received less attention, compared to the direct management of honey bee colonies, is the rearing of new honey bee queens. Many factors influencing successful queen breeding remain unknown. In addition to the beekeeper's expertise, external factors such as pollutants and pathogens associated with materials that come into contact with queen rearing must also be considered.

The results of this doctoral thesis can summarily be stated as follows:

- The changes in the spread of the *Nosema* spp. continues, and it can be assumed that *N. ceranae* is in the process of overtaking the place of *N. apis*. It is clear, that *N. ceranae* has become dominant in Latvia over five years. The pesistant nature of nosemosis was also confirmed – after the five-year gap following the first detection, only a few of apiaries were free of the disease.

- American foulbrood continues to be a problem, and it seems that today, this disease in Estonia has a more latent nature, requiring beekeepers to readapt. Hive debris samples analysed with qPCR technique has proven to be a good and reliable method compared to previously used PCR or microbiological analyses. Collecting hive debris is non-destructive for honey bees, easy to sample for beekeepers and might reflect the potential threat of the disease. The spore laods in apiaries were positively correlated with the numbers of neighbouring apiaries as well as total numbers of naighbouring bee colonies, but the size of own apiary had no effect. Surprisingly, the spore load in apiaries tended to be related to the climatic conditions

– the negative correlation was found with the apiary's longitudinal location.

- The translocation of the pesticide tebuconazole between different beekeeping matrices occurs in constantly decreasing rate. Nearly 20% of tebuconazole mixed into the wax of queen cell cups was translocated into the royal jelly, however no residues were found in the larvae nor adult queen bees.

- High doses of *N. apis* both as pure treatment or in mix with *N. ceranae* caused disruption of metamorphosis at different developmental timepoints. Even if the developing queen bees consumed infected royal jelly, the *Nosema* was not detected in the newly hatched queen bees.

PERSPECTIVES FOR FUTURE RESEARCH

Honey bees, beekeeping, and science as a whole are currently undergoing significant changes. In the coming decades, new challenges are likely to arise, such as the northward movement of the Asian hornet and the *Tropilaelaps* mite. These developments demand that beekeepers adapt and strengthen their capacity for cooperation -both with government sectors and among themselves.

Stress factors affecting honey bees are evolving, requiring adaptation from both beekeepers and bees. Climate change is altering the dynamics of stress factors, including the movement or extinction of pathogens. For instance, the competition between *N. ceranae* and *N. apis*, along with the impact of weather on the spread, viability, and virulence of American foulbrood, highlights the need for focused research. As the vegetation period for plants changes and ecosystems remain in flux, it is essential to develop concrete, sector-specific strategies to ensure sustainable beekeeping practices.

Improving the efficiency of preventive measures is equally crucial. This includes enhancing laboratory analysis capabilities, ensuring adequate supplies, and increasing the competence of analysts. These steps will help ensure that beekeepers can receive swift, affordable, and accurate diagnoses when problems arise. Moreover, the development of new medications and control measures, along with obtaining national approvals for their use, is vital for future preparedness.

Ongoing research should address these challenges by focusing on effective management strategies and treatments that support honey bee health. Such efforts are essential for safeguarding both sustainable beekeeping and global food security.

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SUMMARY IN ESTONIAN

MESINDUSE JÄTKUSUUTLIKKUST MÕJUTAVAD FAKTORID: PATOGEENIDE LEVIK JA DIAGNOSTIKA, MESILASEMADE ARETUS

Sissejuhatus

Tolmeldajate arvukuse märkimisväärsest vähenemisest on teateid kogu maailmas. Nende vähenemisele aitavad kaasa haigused, parasiidid, kehv toitumine, pestitsiidid, elupaikade kadu ja kliimamuutused ning nende tegurite kombinatsioonid. Meemesilane (*Apis mellifera* Linnaeus) mängib tolmeldajana olulist rolli maailma majanduses ja ökosüsteemis, aidates kaasa taimede mitmekesisusele ja toidujulgeolekule. Kuigi suudetakse tolmeldada ligikaudu 15% kultuurtaimedest, on märkimisväärne, et just majandatavate mesilasperede arv on viimastel aastakümnetel taas kasvama hakanud, leevendades mõnevõrra tolmeldajate arvukuse vähenemist. Meemesilasperede hulk ning mesilaste arvukus sõltub suuresti mesinike valmisolekust tootmist arendada, mis põhiliselt sõltub aga mee turustamise võimalustest. Kasvav inflatsioon, suurenenud majanduskulud ja igal aastal Euroopa Liitu imporditavad sajad tuhanded tonnid odavat võltsitud mett mõjutavad märkimisväärselt mesinike otsuseid ja mesindussektori üldist heaolu. Säilitamaks kestlikkust seisavad ka mesilaspopulatsioonid silmitsi keeruliste väljakutsetega. Tänapäeva mesindus ongi kõikide nende väljakutsetega pidevas võitluses. Selleks, et mesindus oleks jätkusuutlik, peavad mesilaspered püsima terved ja mesinikud omama professionaalseid teadmisi riskiteguritest. Mitmeid mesilashaigusi ei olegi laboratoorsete testideta võimalik tuvastada, kuna need võivad olla pikka aega latentsel kujul organismis. Sümptomite puudumisel ei ole võimalik tavapäraste patogeenide tuvastamise meetoditega teha sellistes peredes ulatuslikku seiret ega hinnata haigustekitajate esinemist. Üldjuhul jääb mesinikel puudu ka teadmistest tegurite kohta, mis mõjutavad haiguste püsimist ajas ja nende jaotumist ruumilisel skaalal. Lisaks sellele teatakse vähe ka neist tegureist, mis põhjustavad mesilasperede emata jäämist nn "loomulikul" teel, eriti kevadel, kui mesilasemasid vabalt saada ei ole.

Meemesilasel on kõige levinumaks haiguseks varroos, mida põhjustab lest *Varroa destructor* (Anderson ja Trueman). Lisaks füüsilise keha otsesele kahjule, toimib see ektoparasiit ka erinevate viiruste vektorina.

Mesilaste tervist ohustavateks haigusteks on ka nosemoos, mida põhjustavad pisieoseliste (*Microsporidia*) seltsi kuuluvad *Nosema* liigid ja Ameerika haudmemädanik, mille põhjustaja on gram-positiivne bakter *Paenibacillus larvae*. Nende ohtlike haiguste varajase diagnoosimise ja tõrje on mesinike jaoks äärmiselt oluline, aga äärmiselt keeruline ülesanne. Neist probleemidest lähtuti antud töö kavandamisel.

Töö eesmärgid ja hüpoteesid:

1. Selleks, et määrata nosemoosi püsimist ja selle põhjustajate *Nosema apis* ning *N. ceranae* levikut Eestis ja Lätis, hinnata taaskord mesilaid, mis olid 2013. aasta vaatlustel olnud nakatunud (**Artikkel I**).

   H1.1: *Nosema* liikide levikut, nakatumise taset ja esinemissagedust mõjutavad piirkondlikud tegurid.
   H1.2: Nosemoosist kui väga püsivast haigusest on raske vabaneda, isegi kui mesinikud teavad, et mesilaspered on nakatunud.

2. Hinnata Ameerika haudmemädaniku tekitaja *P. larvae* levikut ja eostega saastatuse taset Eesti mesilates ning selgitada tegureid, mis mõjutavad selle patogeeni levikut (**Artikkel II**).

   H2.1: Patogeeni *P. larvae* tuvastamine langetisest, eriti qPCR tehnoloogia abil toimib ennustava tööriistana mesilasperede tervise hindamisel ja Ameerika haudmemädaniku varajasel avastamisel, võimaldades potentsiaalselt vähendada haiguspuhangute esinemissagedust ja raskust.
   H2.2: Kõige olulisem tegur *P. larvae* eoste levikul Eesti mesilates on mesilasperede tihedus, kusjuures rolli mängivad ka konkreetsed mesila omadused: suurus, teiste mesilate lähedus ja arvukus.

3. Selgitada, kas ja kuidas rasvlahustuv laia toimespektriga süsteemne fungitsiid tebukonasool liigub läbi erinevate mesitaru maatriksite (**Artikkel III**).

   H3.1: Tebukonasool võib mesitarus liikuda erinevate maatriksite vahel.
   H3.2: Kuigi mesilasema peamine toit on mesilasema toitepiim, mis on tõenäoliselt vähem saastunud, võib pidev kokkupuude saastunud vahaga ohustada tema tervist.

4. Määrata, kas ja kuidas pisieoseliste *Nosema* liigid mesilasema toitepiimas mõjutavad mesilasema arengut munast kuni valmikuni (**Artikkel IV**).

   H4.1: Patogeensed pisieoselised mesilasema toitepiimas mõjutavad mesilasema arengut.
   H4.2: Pisieoselised *N. apis ning N. ceranae* jõuavad vastkoorunud mesilasemasse nakatunud toitepiima kaudu.

Materjal ja metoodika

Nosemoosi tekitavate *Nosema* liikidega seotud uuringud viidi läbi 2017. aastal Eestis ja 2018. aastal Lätis (**Artikkel I**). Selleks valiti mesilad, kus oli varem EPILOBEE uuringus (2012–2014) tuvastatud *Nosema* esinemine. Eestist võeti 30 ja Lätis 60 proovi. Katse käigus võeti igast mesilast 60 korjemesilast. Proovid võeti kevadel enne suve- ja talvemesilaste vahetumise algust, kuna sel perioodil suureneb *Nosema* eoste (spooride) tuvastamise tõenäosus. Liikide määramisel kasutati DNA eraldamiseks multiplex PCR (M-PCR) tehnoloogiat. Proovides hinnati eoste hulka, kasutades voolutsütomeetriat (BD Accuri C6), mis suudab eristada ka eoste suurusi ja liike.

Ameerika haudmemädaniku tekitaja *P. larvae* uuring (**Artikkel II**) viidi läbi 2022. aasta kevadel, so ajal kui mesinikud, selleks et vähendada patogeenide levikut, vahetavad mesilasperede põhjasid. Proovid koguti kohalike usaldusmesinike abiga, kes olid eelnevalt koolitatud järgima kindlaid proovivõtuprotokolle, 13. Eesti maakonnast. Igast tarust koguti 500 g materjali, st surnuid mesilasi ja mesilasvaha jääke. Proovid külmutati ja saadeti Ameerika haudmemädaniku määramiseks Eesti Maaülikooli Taimetervise õppetooli laborisse.

P. larvae tuvastamiseks kasutati qPCR-meetodit, mille standardi loomiseks oli kasutatud *P. larvae* ATCC 9545 tüve.

Uuring fungitsiidi toimeaine tebukonasool mõju kohta mesilasema arengule viidi läbi 2020. aastal (**Artikkel III**). Katses kasutati kohalikku mahemesindusvaha, kuhu lisati tebukonasooli (kontsentratsiooniga 412 μg kg^{-1}). Töödeldud vahast valmistati spetsiaalse spaatli abil kupud, kuhu siirdati ühe- kuni kahepäevased vastsed, mis seejärel asetati ülitugevasse ammperesse. Katse viidi läbi juulis, Eesti meevõtuhooaja

ajal. Lähimad põllukultuurid asusid katsest 5,4 km kaugusel, vähendades väliste pestitsiidiallikate mõju. Pestitsiidijääke mesilasema vaklades ning toitepiimas hinnati BIOR Toiduohutuse, Loomatervise ja Keskkonna Instituudis. Analüüs viidi läbi UHPLC-MS/MS meetodiga, kasutades Ultimate 3000 kõrge jõudlusega vedelikkromatograafi (Thermo Scientific, MA, USA).

Katse kahe erineva pisieoselise liigi *N. apis* ja *N. ceranae* mõju hindamiseks mesilasemade arengule, viidi läbi 2021. aastal (**Artikkel IV**). Patogeenide eoste saamiseks kasutati nakatunud töölismesilasi. Vähendamaks pestitsiidide jääkide võimalikke mõjusid katse tulemustele, kasutati kuppude valmistamiseks kohaliku tootja orgaanilist mesilasvaha. Liikide tuvastamine toimus multiplex PCR (M-PCR) meetodiga. Pärast liikide määramist spoorid tsentrifuugiti, saavutades umbes 85% puhtuse. Jääkide eraldamiseks viidi suspensioon läbi 10-mikronilise filtri. Spooride arvu määramiseks kasutati voolutsütomeetrit (BD Accuri C6).

Tulemused ja arutelu

- Vaatlustest selgus, et kahe erineva *Nosema* liigi levik ning arvukuse vahekord on jätkuvalt muutuse trendil. Tööst järeldub, et ka külmema kliimaga piirkondades on hakanud liik *N. ceranae* domineerima ja on asendamas varasemalt arvukama *N. apis* liigiga. Tulemustest selgus ka, et *N. ceranae* on viimase viie aasta jooksul muutunud just Lätis domineerivaks, kuid kasvav trend on ka Eestis. Lisaks täheldasime, et nakatuse tase oli kõrgem neis proovides, mis olid nakatunud mõlema patogeeni tüvega.

- Ameerika haudmemädanik on Eestis kasvavaks probleemiks ja tundub, et haigus on varasemast varjatuma iseloomuga, mis sunnib mesinikke kohanduma. Paljudes mesilates leiti *P. larvae* eoseid, kuid enamasti oli haigestumus väga madalal tasemel. Võrreldes varem tuvastamiseks kasutatud meetoditega, osutus antud töös just varjatud leviku kindlaks määramistel heaks ja usaldusväärseks tundlik qPCR analüütiline meetod. Nakkuse varane tuvastamine võib kaasa aidata haiguspuhangute lokaliseerimisele. Lisaks täheldasime, et *P. larvae* levik on seotud nii klimaatiliste tingimuste kui ka naabruses olevate mesilasperede hulgaga.

- Mesinduses kasutatavad materjalid, aga ka mesilaste toit võivad sisaldada saasteaineid, mis võivad edasi kanduda tarudesse, sealhulgas arenevale haudmele. Uuringust selgus, et esialgu vahale lisatud rasvlahustuva tebukonasooli kogus vähenes edasikandumisel märkimisväärselt – ainult väike osa algsest saasteainest jõudis toitepiima. Arenevas vaglas ega ka koorunud mesilasemades seda ainet ei tuvastatud. Sellest järeldub, et oma lipofiilsete omaduste tõttu koguneb tebukonasool küll mesilasvahasse, kuid selle edasikandumine teistesse mesilasperesüsteemidesse on minimaalne.

- Mesilasemade arenguaegne kokkupuude pisieoselistega (*Nosema* sp.) põhjustas mesilasemadel arenguhäireid väga minimaalses ulatuses. Samas ei tuvastatud *Nosema* eoseid vastkoorunud mesilasemades, kuigi nad olid toitunud nakatatud toitepiimast. Nosemoosi mõjusid mesilaspere erinevatele liikmetele tuleb tuvastada kindlasti edaspidistes uuringutes.

Kokkuvõte

Mesilaste tervis ja mesinduse jätkusuutlikkus sõltub paljudest keerukatest teguritest. Traditsioonilistest epidemioloogilistest uurimisküsimustest kaugemale minnes võib patogeenide varajane avastamine – enne asjakohaste haiguste puhkemist – oluliselt aidata kaasa tervete mesilasperede haldamisele. Uuenduslikud analüütilised meetodid ei paranda mitte ainult analüüsi tulemuste täpsust, vaid tõstatavad ka uusi küsimusi latentsete eoste lubatud arvu kohta tarudes, patogeenide levikut mõjutavate tegurite kohta ning jätkuvaid arutelusid mesinikule kättesaadavate ennetusvahendite üle isegi siis, kui nad on teadlikud võimalike ohtude olemasolust mesilasperedele.

Vähem tähelepanu on saanud aspekt, mis on seotud uute mesilasemade kasvatamisega, võrreldes mesilasperede otsese haldamisega. Paljud edukat emakasvatust mõjutavad tegurid on endiselt teadmata. Lisaks mesindaja oskustele tuleb arvestada ka välisteguritega, näiteks saasteainete ja patogeenidega, mis on seotud materjalidega, mis puutuvad kokku emakasvatusega.

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Article

Variation in the Distribution of *Nosema* Species in Honeybees (*Apis mellifera* Linnaeus) between the Neighboring Countries Estonia and Latvia

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Abstract: The unicellular spore-forming parasites *Nosema apis* and *Nosema ceranae* are considered to be one of the causes of increased honey bee mortality in recent years. These pathogens attack their honey bee hosts through their gut, causing changes in behavioral stress responses and possibly resulting in decreased honey yield and increased honey bee mortality. The present study aimed to determine the prevalence of *Nosema* spp. (nosemosis) in Estonia and Latvia, as well as the persistence of the disease in previously infected hives. Currently, *N. ceranae* is considered the most virulent species and is predominant worldwide. However, in some regions, usually with colder climates, *N. apis* is still prevalent. To achieve better disease control, it is important to determine the species distribution. For this purpose, we selected 30 apiaries in Estonia and 60 in Latvia that were positive for *Nosema* spp. in the EPILOBEE (2012–2014) study, which was 5 years prior to the present study. The results show that, while both species are present in Estonia and Latvia, *N. apis* is dominant in Estonia (43%), and *N. ceranae* is dominant in Latvia (47%). We also found that the pathogens are very persistent, since 5 years later, only 33% of infected apiaries in Estonia and 20% of infected apiaries in Latvia, we could not detect any pathogens at the time of sampling.

Keywords: *Apis mellifera* L.; *Nosema ceranae*; *Nosema apis*; unicellular; pathogens

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1. Introduction

The biggest challenge for beekeepers, from both an economic and ecological perspective, is limiting winter mortality, which means that the honey bee colonies must be healthy. In recent years, winter mortality has been relatively high, which has led to collaboration between scientists and beekeepers to determine the causes [1]. Proposed explanations include increased environmental pressures due to changes in land use [2], the presence of pesticide residues in nectar and pollen [3,4], and changes in the distribution of parasites and pathogens [5]. In northern countries such as Estonia, temperatures have increased and winters are milder, and in the last 10 years, scientists have observed temperature-related changes in insect populations. For example, the typically univoltine Colorado potato beetle can now produce two viable generations in warmer years [6].

Chronic exposure to stressors can cause disorders of the honey bee immune system [7]. One of these stressors is acute *Nosema* infection, which often causes no symptoms, but can decrease honey bee immunity [8], which in turn increases the risk of mortality. Nosemosis is a honeybee disease caused by the unicellular spore-forming fungal parasites *Nosema apis* Zander [9] and *N. ceranae* Fries et al. [10] (division *Microsporidia*) [11]. *N. apis* was thought

to be the only cause of nosemosis in the honey bee *Apis mellifera* L. until 2005, when *N. ceranae* was first described in *A. mellifera* L. in Taiwan [12]. The first *N. ceranae* infection was also found in Spain at the same time [13].

N. ceranae has replaced *N. apis* in many countries [14,15]; it has become predominant in Argentina [16], central Italy [17], Croatia [18], Serbia [19], and South-Germany [20]. *N. ceranae* is also dominant in more northern countries, such as Lithuania and Finland [21,22]. Essentially, *N. ceranae* has spread throughout the world in a short time.

Nosemosis damages the tissues in the honey bee midgut, is energy-consuming for the host, causes changes in behavioral stress responses, and may reduce the life span of the host [23]. Both *N. ceranae* and *N. apis* affect the epithelial cells of the gut, but it has long been thought that *N. ceranae* can also exist in the hypopharyngeal glands, salivary glands, Malpighian tubules, and body fat [24]. However, recently, it was shown that *N. ceranae* has a specific tropism for the epithelial gut, as this was only tissue invaded by this parasite [25]. Despite their generally similar descriptions, these two species affect honey bees differently. *N. apis* outbreaks occur mostly during the springtime and tend to be less severe, while *N. ceranae* outbreaks are detected during the entire period of active colony growth and can cause gradual fading [26]. The hidden course of the disease is also one of the reasons why it is difficult to eliminate. Furthermore, in Europe, no veterinary drugs are registered to control it, because these fungi have developed resistance to antibiotics, which are now ineffective and can leave toxic residues in the hive. The antibiotic fumagillin has been used in beekeeping and has been shown to be effective against nosemosis. However, fumagillin is fairly toxic, can cause chromosomal aberrations, and is carcinogenic to human consumers of honey bee products [27]. Thymol has shown good results in laboratory studies [28,29], and good results against *Nosema* have also been shown for various nutraceutical and immunostimulatory compounds. However, further research is needed in this area [30]. European beekeepers use different measures to control *Nosema* infection. Most of these are hygienic management techniques, but selecting and replacing infected colonies and changing the queen may also help mitigate the infection [31].

The symptoms of nosemosis can vary and can be inconspicuous. The two *Nosema* species are distinguished according to their clinical pattern: *N. apis* causes nosemosis type A, and *N. ceranae* causes nosemosis type C [32]. The latter is more problematic because there is no specific clinical picture. However, the disease might lead to a decrease in honey production and may contribute to mortality [33].

The European EPILOBEE project, which was conducted in 2012–2014, mapped the spread of honey bee viruses and parasites in the member states of the European Union [1]. During the project, 197 samples were taken from hives with clinical symptoms of nosemosis in Estonia in 2012, and 30 of these were positive, and 194 samples were collected from hives in Latvia in 2013, and 60 of these were positive. The persistent nature of the disease, difficulties in self-diagnosis, and variation in the clinical symptoms of the disease caused by the two different species resulted in the need to repeat the survey. Therefore, we aimed to resample previously *Nosema*-positive apiaries to assess the persistence of the disease and determine the distribution of *N. apis* and *N. ceranae* in Estonia and Latvia.

2. Materials and Methods

2.1. The Geographical Location of Apiaries and Honey Bee Sampling

This study was undertaken in Estonia (2017) and Latvia (2018). Apiaries that had previously tested positive for nosemosis (EPILOBEE, 2012–2014), including 30 apiaries (1 apiary = 1 sample) in Estonia and 60 apiaries in Latvia, were resampled. Samples were collected before the major turnover from winter to summer bees (May) to obtain the largest number of spores per bee. In the sampling years, the spring was rather cold, and colonies were just starting their development. For each sample, 60 forage bees were collected from the flying boards of 2–4 neighboring hives using a portable vacuum device. The samples were placed in plastic tubes, cooled immediately for transportation, and frozen at −20 °C until laboratory analyses.

2.2. DNA Extraction and Analysis

Twenty worker honey bees were randomly selected from each sample, and their abdomens were removed using a sterile disposable scalpel. The abdomens were pooled and placed in a 15 × 28.5 cm Universal Extraction Bag (Bioreba). Then, 3 mL of ddH_2O was added to facilitate homogenization using a hand homogenizer. Approximately 800 µL of the suspension was collected for DNA extraction.

DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. A multiplex PCR (M-PCR) assay was performed to identify the *Nosema* species using 2 µL of DNA and primers as described by Fries et al. (2013) [26] (Table 1). The PCR program consisted of a 2-min initial denaturation at 95 °C, followed by 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 57 °C, and elongation for 1 min at 72 °C, with a final elongation step at 72 °C for 5 min. The M-PCR products were visualized on a 2% agarose gel.

Table 1. Primers used to detect *Nosema ceranae* and *Nosema apis*.

Name	Primer Sequence	Fragment Size (bp)	Specificity
Mn*Ceranae*-F	5′−CGTTAAAGTGTAGATAAGATGTT−3′	143	*N. ceranae*
Mn*Apis*-F	5′−GCATGTCTTTGACGTACTATG−3′	224	*N. apis*
Muniv-R	5′−GACTTAGTAGCCGTCTCTC−3′		

2.3. Flow Cytometry and Spore Counting

A flow cytometer (BD Accuri C6) was used to determine the number of spores per honey bee, and, in cases of a mixed infection, the total number of spores are presented. An aliquot (50 µL) of the suspension generated for DNA isolation was diluted with 1 mL of ddH_2O, and 50 µL of the dilution was used for the spore counting analysis. A disposable sieve (10 µm) was used to remove debris. To determine the number of spores per honey bee, the following formula was used: $x = \frac{3000 \times 1050}{20 \times 50 \times 50} \times SA$ (spores in the sample). The formula is explained as follows: 3000 (3000 µL of water added to facilitate homogenization), 1050 (final mixture from which 50 µL of spore suspension was removed was diluted with 1000 µL of water), 20 (number of bee abdomens used), 50 (50 µL of spore suspension used in the dilution), and 50 (50 µL of the filtered spore suspension was loaded into the flow cytometer for spore counting). The accuracy of the results was compared to the microscopic spore counts from five samples of each infected species. There was no significant difference between the results [KW-H = 0.06, $p = 0.73$].

2.4. Statistics

Data were processed using TIBCO Statistica® 13.3.0. The Kruskal-Wallis test was used to assess the statistical differences in spore number per honey bee in Estonia and Latvia and confirm the accuracy of the flow cytometry results. The chi-square test, which compares the differences in shares of subdivisions, was used to examine the statistical significance of differences between *N. ceranae* and *N. apis* distribution changes in Latvia. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Prevalence of N. apis and N. ceranae

Multiplex PCR showed that both Nosema species were present in Estonia and Latvia, either separately or in co-infections. The species distribution varied between the two countries. *N. apis* was the more prevalent species in Estonia, while *N. ceranae* was more prevalent in Latvia at the time of sampling. Among the 30 sampled apiaries in Estonia, 17% were positive for *N. ceranae*, 43% were positive for *N. apis*, and 7% were co-infected. In the remaining 33% of sampled apiaries, *Nosema* was no longer detected. In Latvia (n = 60), the results were almost the opposite: 47% of the samples were positive for *N. ceranae*, 15% were positive for *N. apis*, and 18% were co-infected. In

the remaining 20% of sampled apiaries, *Nosema* was no longer detected in the sampling period (Figure 1).

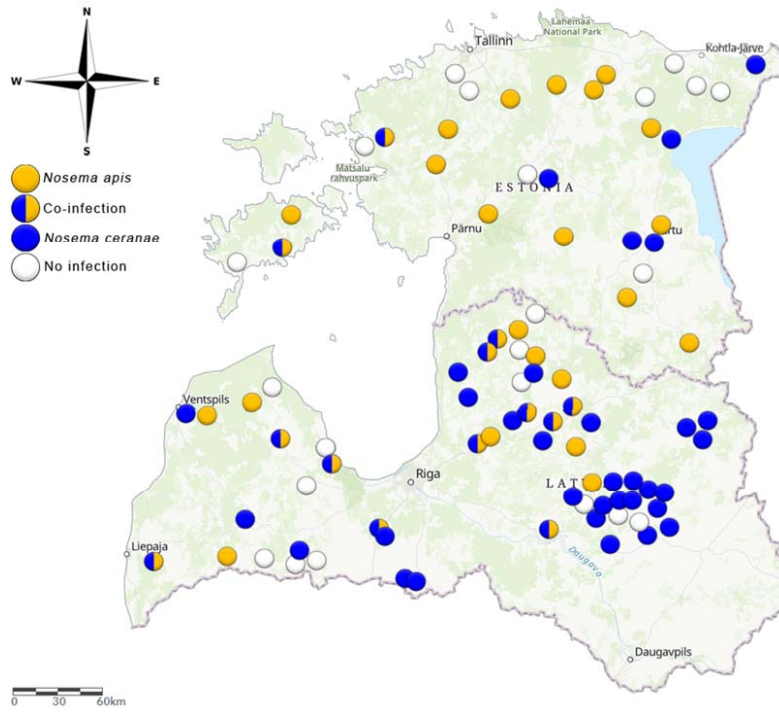

Figure 1. Prevalence of nosemosis causative agents (*Nosema ceranae* and *Nosema apis*) in Estonia in 2017 (May) and Latvia in 2018 (May) (ArcGis online).

3.2. Spore Quantity

The number of spores per honey bee was the highest when both species were present. The median count (spores per honey bee) in co-infections was approximately 12 million in Estonia and approximately 9 million in Latvia. In single species infections, the median spore count for *N. apis* was approximately 6 million, while that for *N. ceranae* was approximately 4 million in Estonia. There were no statistically significant differences in spore counts between *Nosema* species or mixed infections (Figure 2). In Latvia, the median spore count for *N. apis* was approximately 1.7 million and that for *N. ceranae* was approximately 2.4 million spores per honey bee (Figure 2), which was significantly lower than that in co-infections.

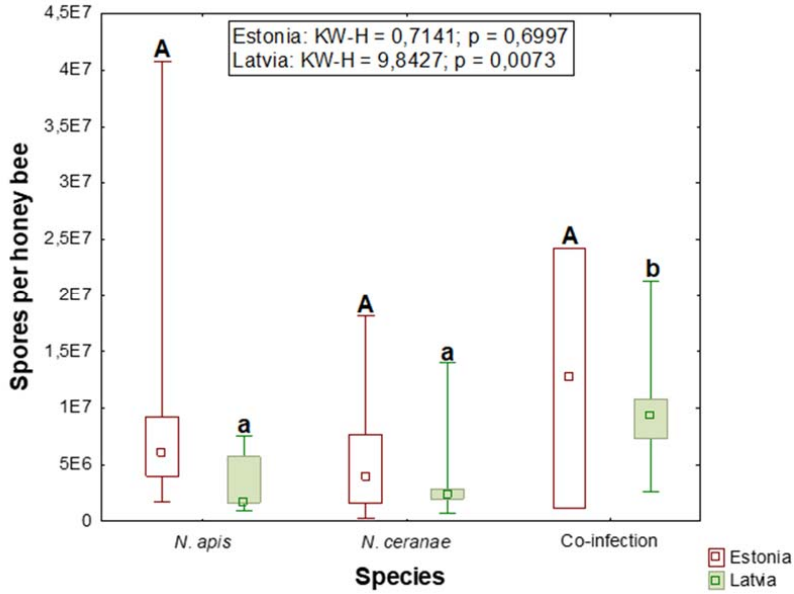

Figure 2. Bar graphs depicting the numbers of *Nosema* spores per honey bee in samples from Estonian and Latvian apiaries, as measured using flow cytometry. Error bars are the minimums and maximums. Columns with different letters indicate significant differences. Statistical differences were calculated by the Kruskal-Wallis test, with significance set at $p < 0.05$.

3.3. Species Distribution Changes in Latvia

A significant change in the distribution of *Nosema* species in Latvia was observed ($\chi^2 = 35.71$, $p < 0.00001$). In 2013, 65% of collected samples were co-infected (unpublished data from Latvian Beekeepers Association), 15% were infected with *N. apis*, and 20% were infected with *N. ceranae* (Figure 3a). However, in 2018, 47% of apiaries were infected with *N. ceranae*, 18% were infected with both species, and 15% were infected with *N. apis*; 20% of the apiaries in 2018 were those in which we could not find any *Nosema* (Figure 3b).

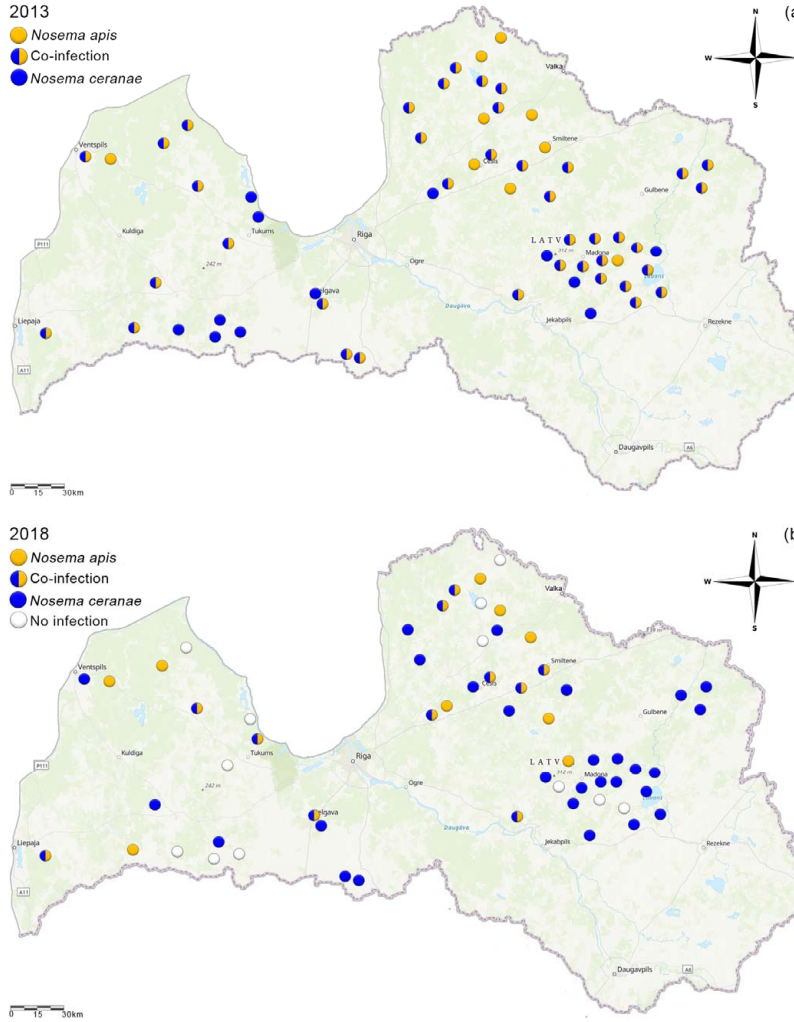

Figure 3. *Nosema* species prevalence in Latvia in 2013 (May) (**a**) and 2018 (May) (**b**) (ArcGis online).

4. Discussion

Our study showed that both *Nosema* species causing nosemosis are present in Estonia and Latvia. The causative agents were found for both single infections and co-infections.

Interestingly, the species composition varied greatly between the two neighboring countries at the time of sampling. *N. apis* was the most abundant species in Estonia, while *N. ceranae* was the most abundant species in Latvia. We observed similar spore counts for single-species infections of both species, whereas co-infected colonies had higher spore counts.

Since its first discovery in *A. mellifera* in Taiwan in 2005 [10], *N. ceranae* has become dominant in *Nosema*-infected apiaries. Over the last few decades, the original pathogenic species, *N. apis*, has been displaced by this more aggressive pathogen. Although for a long time, these two species were thought to be host species specific [13]. It is now clear that these species can exist either individually or together. Still, there is a question of whether a co-infection is more damaging to honey bees than a single infection. In cage studies where the spores of the two pathogens were fed to infection-free honey bees, both *Nosema* spp. were found to be virulent. This was especially true for *N. ceranae* and a mixture of *N. ceranae* and *N. apis*, as both groups of honey bees showed decreased longevity and viability [33,34]. This decrease in survival may result from the reduced defence mechanisms of the honey bees which makes the host more vulnerable to various external factors [35]. In addition, *N. ceranae* tends to be more aggressive during the rapid development phase of honey bee colonies, which in turn contributes to the increased number of spores [15]. In our study, the median number of spores per worker bee ranged from 1.6 to 14 million. A very similar result was shown by Odemer et al. [36]. Although they infected bees artificially and reported cross-infection of their *N. apis* honey bees. This could be explained by the fact that the artificial *Nosema* infection relies on viable spore material. Maybe the spores used for the *N. apis* infection were somehow of lower viability than in a natural setting and expressed therefore lesser spores in the infected honey bees. Additionally, at the end of the paper, they mention the fact that laboratory or semi-laboratory results depend on many factors which may affect the results. It is difficult to draw convincing conclusions about the correlation between high spore counts and colony losses [37] because in field and semi-field studies it is difficult to exclude the possible co-effects of other factors. Another question is whether an infection with these pathogens can lead to colony losses. Exposure to various stressors (pesticides, parasites, etc.) can significantly increase mortality. For example, exposure to various neonicotinoids (e.g., clothianidin and imidacloprid). However, here too, the results diverge. Alaux et al. [38] demonstrated that an interaction between *Nosema* spores and imidacloprid reduced the lifespan of honey bees and neonicotinoid exposure weakened colonies. Odemer et al. [36] demonstrated that by applying neonicotinoid clothianidin in field-relevant sublethal concentrations to free-flying colonies, the neonicotinoid clothianidin did not act synergistically either with *N. apis* or with *N. ceranae*. However, this neonicotinoid is considered to be very toxic to honey bees [39].

Estonia and Latvia are small neighboring countries with similar climates, thus climatic variation is an unlikely cause of the differences in species distribution. It is possible that *N. ceranae* is still increasing its range. This could be investigated by repeating the study after a shorter time and including random hives. A similar increase in the range of *N. ceranae* was also shown by Ostroverkhova et al. [40]; despite large climatic differences in the study region, they were not able to show a climate dependence in the relative spread of these two species. Pacini et al. [16] recorded infections with *N. apis* only in the subtropical regions of Argentina, whereas in the temperate regions, *N. apis* was detected only in co-infected colonies. Conversely, only *N. ceranae* was found in Saudi Arabia [41]. Finally, a study from Mexico showed that *N. apis* was dominant (87%) in an area with a warm climate [42].

Estonia seems to be one of the few countries in the world where *N. apis* is still individually prevalent. In a 4-year study carried out in Lithuania [22] which also looked at the distribution of these pathogens, their proportions were very similar, and neither species was dominant. This result is in contrast to our findings in Estonia and Latvia, where *N. apis* was prevalent in Estonia and *N. ceranae* was prevalent in Latvia. It has reported that *N. ceranae* has become increasingly dominant in Finland since 2006, whereas before that, *N. apis* was dominant [21]. It is possible that the geographical location of Estonia could explain the unique prevalence of *N. apis*, since honey bees cannot cross the Baltic Sea from

Finland, the natural spread of honey bee pathogens could occur only from the south or east. However, *Nosema* can spread with the assistance of people, through the import of infected colonies, small nuclei of colonies or queens. In Estonia, imported queens are widely used. These queens come mainly from Romania and Italy, where *N. ceranae* is a common pathogen [43,44]. However, this does not explain why *N. apis* is more common in Estonia.

As there is no effective cure for *Nosema*, beekeepers need to use uncontaminated equipment in apiaries. The internal temperature of a honey bee colony is always 32–35 °C, which is a favorable temperature for the pathogen. A cold climate could somewhat aid in the fight against *Nosema*. This has led beekeepers to believe that cold storage of beekeeping equipment may kill the spores. However, studies have shown contrary and largely variable results. For example, Ozgor and Keskin [45] showed that *N. ceranae* is still infectious after 1 year at −20 °C. They also found that a milder cold temperature (4 °C) was even more conducive to spore survival. Finally, according to Fenoy et al. [46], there was no significant increase in spore mortality within a few hours or after a few months when the spores were exposed to a warm (35 °C) or very warm (60 °C) environment. This indicates that sterilization of beekeeping equipment after every usage in infected colonies is important to avoid spreading the spores from one apiary to another.

Our study samples were collected once in the spring when the age distribution of honey bee colonies exchange for younger bees. However, only one sampling date may create a situation where the *Nosema* prevalence may shift during the season and after overwintering. For future studies, we recommend at least three sampling times (spring, summer, autumn) to investigate the seasonality of the two pathogens in areas with colder climates. Moreover, to get an idea of the species distribution, similar samples should be taken over several years. It is still unclear whether the two pathogen species exhibit seasonality over a longer period. Based on previous studies, *N. apis* infection is present predominantly during the springtime. This is because in spring, only old bees that have overwintered in the hive are present, the queen is just beginning to lay eggs, and the laying intensity is low. Old bees are more susceptible to infection, and when nursing the hive, spores spread through the faecal-oral route [47] and reinfect the overwintered worker bees. The latter may also be true for *N. ceranae*. However, several studies have confirmed that it is difficult to find clear links between seasonality and species occurrence. Although spore counts are usually higher in the springtime, they can vary annually and depend on several other factors [19,26,35]. In our experiment, forager bees were collected, which may have affected the study results, because, according to Meana et al. [48], in-hive bees had fewer spore counts than foragers. In the future, in-hive bees should be examined to better describe the extent of the infection in the colony.

5. Conclusions

We conclude that the spread of *N. ceranae* may be lesser in regions with colder climates, but further research is needed. To clarify the threat of nosemosis to honey bees in various regions, we need to understand the co-effects of various stressors on infection severity, as well as how to protect honey bees so that their immune system can more effectively fight these internal pathogens. Additionally, from a long-term perspective, nation-wide and pan-European monitoring programs should cover the spread of *Nosema* more accurately and future research should focus on establishing such networks.

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ORIGINAL RESEARCH ARTICLE

Could hive debris samples and qPCR ease the investigation of factors influencing *Paenibacillus larvae* spore loads?

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ABSTRACT

American foulbrood (AFB) is a serious disease caused by the spore-forming bacterium *Paenibacillus larvae* which can cause honey bee colony death within one season. Determining the number of *P. larvae* spores present in honey bee colonies is useful for users for assessing and mitigating AFB risks and can be performed in various bee matrices, including hive debris. The quantitative polymerase chain reaction (qPCR) method is a valuable tool for defining the *P. larvae* load in beehive matrices. We aimed to analyse hive debris from several Estonian apiaries, using qPCR methodology, and to find correlations between *P. larvae* spore number and characteristics of the sampling sites, such as apiary geographical location and dimensions, closeness to other apiaries, and number of apiaries and hives within a 7 km radius. Of the analysed samples, 52.5% were positive for *P. larvae*, and we observed high variability in spore loads. A positive correlation was found between *P. larvae* spore levels and the number of surrounding apiaries and apiary location. Applying *P. larvae*-targeted qPCR to hive debris could allow the estimation of AFB risk in areas with high apiary density.

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Introduction

Honey bee colonies (*Apis mellifera*) are threatened worldwide by the infectious American foulbrood (AFB) disease, which is caused by the gram-positive spore-forming bacterium *Paenibacillus larvae* (Hansen & Brødsgaard, 1999). AFB is a brood disease that exclusively attacks larvae and is one of the most acute bacterial diseases to cause death in honey bees. The disease vectors are nurse bees that carry spores around the hive. Spores enter the digestive system of the larvae through food. The larvae are most at risk at approximately 36 h old (Genersch, 2010). One of the signs that should alarm the beekeeper is an unusual odour, often compared to the smell of a carpenter or wood glue. Additionally, the brood pattern may be patchy. The larvae die soon after their cell is capped; therefore, if a stick is inserted through the capping and a sticky mass adheres to it, AFB is diagnosed. Since bee health can vary or be affected by stress factors – the clinical presentation varies widely and the clinical signs may not always manifest (Matović et al., 2023). Despite the large number of scientific works on AFB, only a few have explored novel solutions for AFB control that are not yet implemented in beekeeping practice (Locke et al., 2019; Matović et al., 2023). Burning infected colonies and inventories is still the most effective but remains a costly remedy for apiary recovery. Burning is only mandatory in certain countries (Datta et al., 2013). Estonian practices for AFB detection and treatment follow EU regulations 2016/429 (Regulation—2016/429, 2016) and thus the use of antibiotics is forbidden. However, there is a grey area, and burning AFB-infected colonies is optional rather than mandatory. In addition, no compensation is provided to the beekeepers of the burned colonies.

Another issue related to AFB is its varying manifestations in honey bee colonies. There is no threshold number of *P. larvae* spores detected in the hive matrices that indicates the clinical manifestation of the disease. Various works have proposed different *P. larvae* spore numbers causing clinical symptoms (Gende et al., 2011; Hansen & Brødsgaard, 1999). The manifestation of an AFB outbreak depends on the health status of the bee colony and the presence or absence of other features (Stephan et al., 2020). A similar phenomenon has been observed in honey bee colony tolerance to different pesticides in various environments with different food availability (Colin et al., 2019). Various publications have

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indicated that a range of *P. larvae* spores that are potentially detrimental to honey bee colonies can be inferred. A ratio of 228–3000 *P. larvae* spores per worker bee may induce clinical symptoms in honey bee colonies (Gende et al., 2011; Stephan et al., 2020). However, even lower *P. larvae* spore counts have been shown to reduce bee colony lifespan, especially combined with other stress factors, such as synthetic pesticides (López et al., 2017). Many researchers agree that bees face several simultaneous stress factors and assessing the exact impact of a single factor is difficult; thus, bee health problems must be approached in a complex way.

Accurate determination of *P. larvae* spore load in a bee colony can greatly aid the control and prevention of potential outbreaks. To date, plate count methodology has been used to assess the presence and amount of *P. larvae* spores. Nevertheless, the plate count methodology must be reassessed because of low and inconsistent germination rates of *P. larvae* in culture media (Crudele et al., 2020), producing inaccurate results. Although standard polymerase chain reaction (PCR) methods do not allow spore abundance estimation, the quantitative polymerase chain reaction (qPCR) technology has been proposed as a faster and more reliable tool for *P. larvae* detection and quantification (Rossi et al., 2018). Several matrices have been used to detect the *P. larvae* spores in honey bee colonies. For example, bees, wax, pollen, and hive debris can be analysed for AFB detection (Adjlane et al., 2014; Wilhelm et al., 2023). Using hive debris collection (easy to collect, minimal colony disturbance) for *P. larvae* spore detection provides a reliable year-round historical reflection of accumulated *P. larvae* spores in honey bee colonies, and the analysis of debris for *P. larvae* spores in spring allows beekeepers to detect potential threats early in the season when brood presence is low. In addition to hive debris, qPCR is an effective tool for *P. larvae* spore determination and quantification in honey (Kušar et al., 2021). These screening methods improve predictions of colony health status, and may assist in preventing potential AFB outbreaks.

This study aimed to define the distribution and levels of *P. larvae* spore contamination in Estonian apiaries and determine whether apiary dimensions, density, and distance were correlated with the spread of this pathogen. It was hypothesized that the number of *P. larvae* spores in samples correlates with higher apiary density and the apiary geographical location. Hive debris was used as the sample type because it is an appropriate matrix for determining *P. larvae* spores (Adjlane et al., 2014; Wilhelm et al., 2023; Zabrodski et al., 2022). The foraging radius of honey bees can easily exceed 4 km (Raimets et al., 2020). As the precise foraging distances were unknown, we used the probable maximum country-specific foraging radius. Thus, the honey bee colony density within a 7 km radius of a sampled apiary was considered. Only beekeepers included in the Agricultural Registers and Information Board (PRIA) of Estonia were considered as their honey bee colonies are subject to mandatory registration. The register provides valuable information about honey bee colony density in each area. Beekeepers and farmers can use this information to manage and protect their beehives.

Materials and methods

Sample collection and processing

Hive debris samples ($N = 179$) were collected in the early spring of 2022 from Estonian apiaries in 13 counties. The apiaries examined in this study were selected randomly. The goal was to obtain samples from as many counties as possible to cover most active beekeeping areas in Estonia. The sampling period was in early spring (March); thus, no clinical signs of AFB occurred because of the low amount of brood during this period. Pooled ($N = 137$) and individual ($N = 42$) hive samples were collected. For pooled samples, the number of hives was 2–10 when the apiary had up to 10 hives. A maximum of six pooled samples were collected from larger apiaries. For comparison, hive debris samples were collected from each hive from one small apiary and two large apiaries.

Samples of dry hive debris from the bottom of the hive were placed into 1500 ml boxes using disposable sampling spoons. For each sample, a sampling form was completed, and an unique code was assigned. Within 12 h after the collection, the samples were frozen at −20 °C.

In the laboratory, dead bees were separated from debris using disposable spoons. At least 2 g of debris was packed separately into 50 ml falcon tubes. After the final quality and quantity control, 179 samples were used for the detection and quantification of *P. larvae* using qPCR. Tubes with debris were sent for qPCR analysis in refrigerated transportation to the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale" (IZSAM), Italy.

Determination of the number of surrounding apiaries

The sampled apiaries were located on the map, and then information requests on the number of apiaries and hives within a 7 km radius and the distance to every neighbouring apiary, were sent to the PRIA. The data was used to correlate the number of

determined *P. larvae* spores with the density of the surrounding apiaries.

Bacterial strains and growth conditions

Paenibacillus larvae ATCC 9545 was used to obtain qPCR quantification standards. This strain was streaked onto Sheep Blood Agar (Biolife Italiana, Milan, Italy) and incubated at 37 °C for 2–5 days in the presence of 9% CO_2. Single colonies were streaked onto the same medium and allowed to grow under identical conditions. Biomass from this second culture was dispersed in 10 ml of sterile phosphate-buffered saline (PBS, 8.0 g/L NaCl, 0.2 g/L KH_2PO_4, 2.9 g/L Na_2HPO_4, 0.2 g/L KCl, pH 7.4) and heated at 80 °C for 10 min in a water bath to kill vegetative forms. The spores were harvested by centrifugation at 12.000 rpm for 5 min and washed twice with sterile PBS. Their number was determined by plate count on Sheep Blood Agar and serially diluted to inoculate hive debris sterilised by autoclaving at 121 °C for 15 min to prepare the qPCR quantification standards.

DNA extraction from hive debris samples

The method described by Rossi et al. (2018) was modified and used to detect *P. larvae* in hive debris samples. The modification consisted of the replacement of proteinase K lysis by bead beating. Briefly, 100 mg aliquots of debris were transferred in 2 ml Eppendorf tubes containing 200 µl of T1 buffer (Macherey–Nagel GmbH & Co. KG, Düren, Germany), and 100 mg of sterile glass beads with a diameter of 200 µm (Merck). This mixture was disrupted in a Tissue Lyser II (Qiagen, Hilden, Germany) at 30 Hz for 3 min. Bead-beating was chosen over proteinase K because of its shorter lysis time (3 min compared to 1 h with proteinase K) and lower cost. Bead-beating yielded DNA extraction results comparable to those obtained with proteinase K treatment, as evidenced by the statistical analysis of Ct values in samples artificially contaminated with known quantities of *P. larvae* spores (data not shown). Subsequently, 200 µl of B3 buffer (Macherey–Nagel) was added and thoroughly mixed with the homogenate. The suspension was centrifuged at 12,000 rpm for 10 min, and the supernatant was transferred into a clean Eppendorf tube pre-filled with 210 µl pure ethanol. The mixture was allowed to precipitate for 10 min. The ethanol mixture was passed through a column before washing. The sample was purified using DNA extraction columns according to the instructions of the NucleoSpin Tissue Kit (Macherey–Nagel). The same method was used to prepare qPCR quantification standards for artificially contaminated hive debris samples.

The quantification standards were prepared by extracting triplicate series of hive debris samples inoculated with decimal dilutions of *P. larvae* spores from 10 to 1,000,000 CFU of *P. larvae* spores/g and obtaining amplification and calibration curves as reported in Rossi et al., 2018. This allowed addressing variability in DNA extraction yield. The presence of a standard curve in each experiment allowed quantifying *P. larvae* in samples by using the qPCR machine software as commonly done for absolute quantification in qPCR.

P. larvae detection and quantification by qPCR

Quantitative PCR was carried out in a QuantStudio 5 thermal cycler (Applied Biosystems, Thermofisher Scientific, Rodano, MI, Italy) in 20 µL reactions according to Rossi et al. (2018) using the qPCRBIO SyGreen Mix Separate–ROX qPCR Master Mix (PCR Biosystems, London, UK) with primers PLAup (5′-TTCGGGAGACGCCAGGTTA-3′)/PLAdw (5′-CTTTCATGA CTTCTTCATGCGAAG-3′) used at final concentrations of 0.25 and 0.15 µM, respectively. Calibration curves for *P. larvae* quantification were constructed in each qPCR run using two series of DNA standards represented by DNA extracted from autoclaved hive debris artificially inoculated with decimal dilutions of *P. larvae* ATCC 9545 spores in the range of 1–1,000,000 CFU/g. The quantification of *P. larvae* spores in each sample was provided by QuantStudio 5 Design and Analysis software v1.5.2. Non-specific amplification curves were excluded after melting curve analysis, which gives a melting peak specific for *P. larvae* at 84 ± 1.5 °C, as ascertained by testing the *Paenibacillus* species with the highest sequence similarity in the target region (Rossi et al., 2018).

Statistical analysis

Data was processed using STATISTICA 13. The chi-square test was used to compare the distribution of pooled and single-hive samples in different infection categories (negative, detected, <LOQ: low, high, and very high). The Kruskal–Wallis (KW–H) test was performed to compare the numbers of spores in single hive samples with pooled samples.

Non-paramteric permutational Spearman rank-order correlation was used to assess whether the amount of hives in the apiary or the number of neighbouring beehives and the distances to these were correlated with the number of *P. larvae* spores in the samples. The same test was used to assess the correlation between longitude and latitude and the number of *P. larvae* spores (log values). The

parametric Pearson correlation coefficient and *p*-value are calculated based on linear relationship between the numbers of registered apiaries and proportions of positive samples. To avoid the results from being influenced by an excessive number of samples in the two apiaries where we sampled each hive, we randomly selected six samples for use in these correlations. The correlations were obtained using 154 samples.

Results

The results showed that 52.5% of the analysed samples were positive for *P. larvae*. Most samples showed a low infection rate, with the number of *P. larvae* spores below 10 CFU/g, i.e., Ct values higher than those of the standards corresponding to 10 spores of *P. larvae*. The linear zone of the calibration curve in all PCR runs was at least five orders of magnitude from 100 CFU/g spores to 1,000,000 CFU/g spores. Below 100 CFU/g and above 1,000,000 CFU/g spores, the quantification was not reliable because of the linearity range of the calibration curve. The limit of detection (LOD) of the PCR test and the calibration curves for primers was previously determined by Rossi et al. (2018) as 10 CFU/g spores of *P. larvae* for hive debris. Therefore, samples with a cycle threshold equal to or higher than that corresponding to 10 CFU/g spores in each experiment were classified as detected. According to different ranges of spore numbers obtained from the hive debris samples examined, the quantities of the spore loads were classified, as negative, detected <10 CFU/g, <LOQ (100 CFU/g), low (152–204 CFU/g), high (623–932 CFU/g), and very high (183,000 to >1,000,000 CFU/g). Intermediate ranges of 1000 – 182,000 CFU/g were not determined from samples and thus were not included.

From all the samples tested, 48.2% were negative, 27.1% were positive, but remained under limit of quantification, 9.4% remained under the limit of classification, 5.9% of the samples contained variable number of spores, ranging from the category "low" to "very high." The proportions of negative samples and those where spores were detected at variable levels, as shown in Figure 1, were significantly different (Chi2 = 31.5, $p < 0.001$) between pooled and single hive samples. Figure 2 demonstrates also significantly lower median values of pooled hive samples compared to single hive samples (KW–H (1;93) = 9.40; $p = 0.002$), indicating that the pooled samples dispersed spores from highly contaminated hives.

There was a slight positive but statistically insignificant correlation ($r = 0.32$; $p = 0.29$; $r^2 = 0.10$) between the number of registered beehives in each county and the proportion of *P. larvae* positive samples (Figure 3). The spore load was positively correlated with the number of neighbouring apiaries and the number of beehives within a radius of 7 km around the sampled apiary, and negatively correlated with the apiary's longitudinal location (Table 1). Simultaneously, the number of beehives in the target apiary, distance to the closest neighbour, and average distance to the neighbouring apiaries were not correlated with the spore load in the hive debris samples.

Despite very high spore loads in some hive debris samples during springtime, AFB clinical signs were still not evident in the colonies (Table 2) during the following summer. In the 42 individually sampled bee colonies, which had variable and some had very high spore loads, no AFB outbreaks occurred the following summer, according to beekeeper reports. One colony with a very high spore load faded during summer, i.e., the colony remained very small and subsequent queen failure occurred.

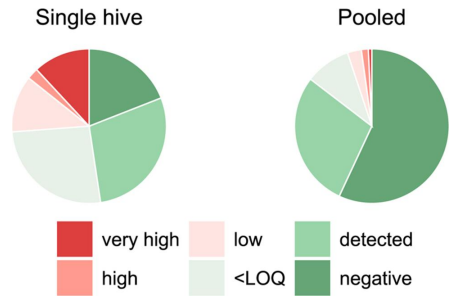

Figure 1. Samples distribution based on the *P. larvae* spore level in pooled (2–10 hives per sample) or single hive samples. Legend: negative, detected <10 CFU/g, <LOQ (100 CFU/g), low (152–204 CFU/g), high (623–932 CFU/g), and very high (183,000 to >1,000,000 CFU/g).

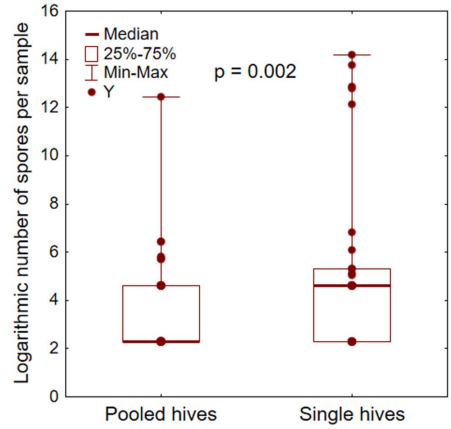

Figure 2. The logarithmic numbers of *P. larvae* spores detected in hive debris and sampled either from pooled (up to 10 hives) or single hives. The difference is significant, $p < 0.05$.

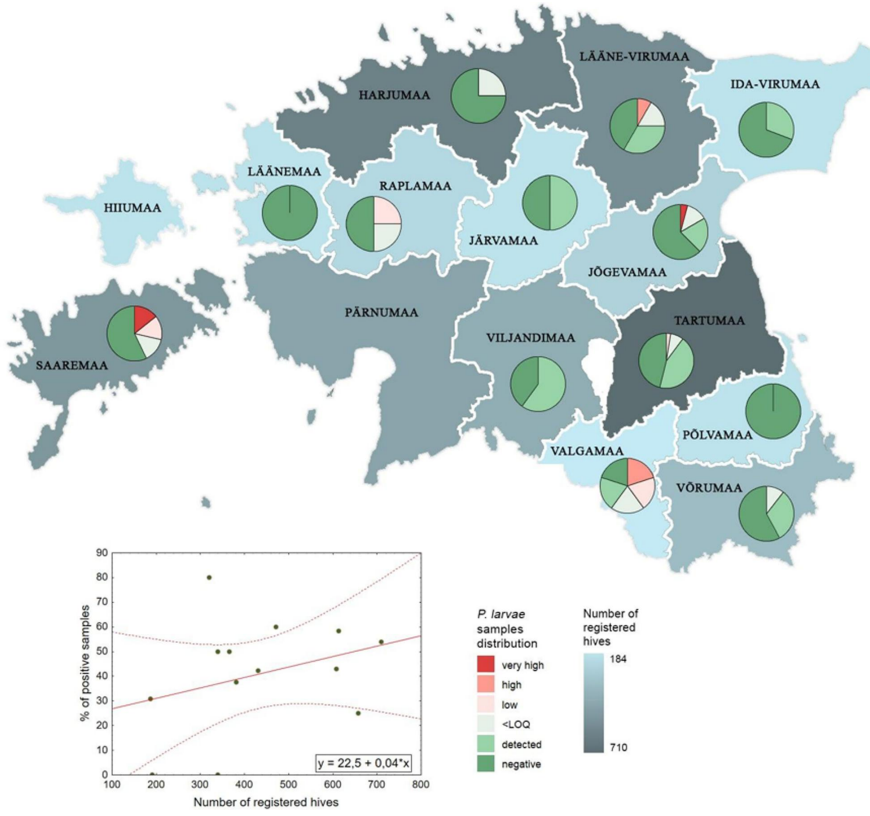

Figure 3. The distributions of samples with various levels of *P. larvae* spores in hive debris samples (green-to-red coloured circles) on the background of numbers of registered beehives in different counties of Estonia (light-blue-grey colour scale). The Pearson correlation equation with linear relationship line and 95% confidence intervals is presented for these two parameters.

Table 1. Spearman rank order correlations between the number of *P. larvae* spores and the numbers of own or neighbouring beehives and distances to these.

	Spearman rank order	*p*
Number of own beehives	0.004	0.964
Number of other apiaries (radius of 7 km)	**0.253**	**0.002**
Number of other beehives (radius of 7 km)	**0.241**	**0.003**
Distance to closest neighbouring apiary	−0.038	0.643
Average distance to apiaries (radius of 7 km)	0.081	0.320
Latitude*	−0.04	0.7334
Longitude*	**−0.411**	**0.0004**

Non-parametric permutational Spearman rank order correlation test was used. The comparisons are considered significant when the difference between groups is greater than or equal to 5%, i.e., $p \leq 0.05$.
*Log (*P. larvae* spores).

Discussion

This study reported the *P. larvae* spore distribution and levels in Estonian apiaries and represents the first study on the distribution of this pathogen within the country. Previously, during the EPILOBEE project (2012–2014), the presence of AFB in some Estonian apiaries was detected based on clinical symptoms (De Graaf et al., 2016).

In this hive debris screening study, approximately half of the samples tested positive for *P. larvae*, but most had low spore loads. Even for very high spore loads, disease outbreaks were not reported by beekeepers. This is not surprising because clinical symptoms can become visible late during infection (Regulation 2010/37, 2010) if a brood is present in the hive. Moreover, the clinical symptoms of AFB are estimated to remain undetected in as many as 25% of bee colonies with high *P. larvae* spore numbers (Bassi et al., 2018; Datta et al., 2013; Jończyk-Matysiak et al., 2020; Stephan et al., 2020). In particular, Bassi et al. (2018) showed that hive debris analyses can provide the best estimate of AFB risk. However, they also reported that not all the hives with the highest levels of *P. larvae*, above 100,000 after winter, developed AFB during spring. Usually, hives are tested when suspicious signs indicate potential AFB, leading to the analysis of suspicious brood combs. Different matrices might hide or accumulate spores variously; thus, there is a need for a scientific understanding of how to interpret the outcomes (Wilhelm et al., 2023).

The qPCR method was preferred for *P. larvae* detection and quantification in this study because of its several advantages over the plate count methodology. Crudele et al. (2020) showed that this technique identified more positive samples compared to the plate count method, and some of these considered negative with plating technique were confirmed as true positives by observing the growth of *P. larvae* under improved growth conditions. This study used qPCR and identified 51.8% of the samples positive. Among all the positive samples more than half were those with *P. larvae* spore numbers below 10 CFU/g, which probably would have been missed by a culture assay. Even the detection of low spore levels can improve the identification of apiaries with increased risk.

Analysing the presence and quantity of *P. larvae* spores in hive matrices could prevent AFB spread more effectively than traditional visual inspections of disease symptoms by beekeepers. Many colonies may hide *P. larvae* spores with relatively few symptomatic cases, escaping detection and continuing to be a source of infection both within a beekeeping operation, between beekeepers (through the sale of bees and equipment), and to feral and managed colonies within flight range through drifting and robbing (Fries et al., 2006). Therefore, routine screening of *P. larvae* spore levels non-destructively sampled from honey bee colonies represents a reliable way to assess the risk of epidemic spread at local, regional, and national scales by unambiguously identifying all

Table 2. Detailed results of three apiaries, where each hive was sampled for the debris analyses for *P. larvae* spores and the beekeepers' subjective estimation of the disease symptoms during summer.

Apiary	Colony size*	Ct	CFU/g	Disease symptoms
1	Normal	>40	0	No
1	Normal	39.00	<10	No
1	Normal	38.33	<10	No
1	Normal	38.76	<10	No
1	Normal	36.95	10–100	No
2	Normal	>40	0	No
2	Normal	>40	0	No
2	Normal	37.78	10–100	No
2	Normal	38.61	<10	No
2	Normal	38.52	<10	No
2	Normal	>40	0	No
2	Normal	37.84	10–100	No
2	Normal	>40	0	No
2	Normal	>40	0	No
2	Normal	38.66	<10	No
2	Normal	38.52	<10	No
2	Normal	>40	0	No
3	Nucleus	37.64	10–100	No
3	Nucleus	37.33	10–100	No
3	Nucleus	37.26	10–100	No
3	Nucleus	38.29	<10	No
3	Nucleus	37.21	10–100	No
3	Nucleus	>40	0	No
3	Nucleus	38.72	<10	No
3	Nucleus	17.20	362,000	No
3	Nucleus	29.54	445	No
3	Nucleus	17.95	183,000	No
3	Nucleus	17.18	391,000	No
3	Nucleus	29.81	152	No
3†	Nucleus	16.40	942,000	No
3	Nucleus	28.21	932	No
3	Nucleus	15.31	>1,000,000	No
3	Nucleus	29.65	204	No
3	Nucleus	37.13	10–100	No
3	Nucleus	37.99	<10	No
3	Nucleus	38.75	<10	No
3	Nucleus	29.72	169	No
3	Nucleus	36.98	10–100	No
3	Nucleus	37.56	10–100	No
3	Nucleus	37.22	10–100	No
3	Nucleus	38.25	<10	No
3	Nucleus	29.65	204	No

*Normal colony: a normal size hive (at least 10 langstroth frames); nucleus colony: hive with up to six langstroth frames.
†Queen failure.

infectious colonies rather than just those presenting detectable symptoms. We suggest that screening apiaries at the national scale early in the season would allow beekeepers timely sanitation before the possible disease outbreak. In Europe, preventive methods are essential as no AFB treatment exists. Screening should consider beehive density rather than regional borders, as the *P. larvae* spore loads correlate with actual beehive densities.

In some countries, AFB is classified as a notifiable disease, and reporting to veterinary authorities is mandatory if symptoms of AFB are discovered in an apiary. To date, the most reliable control method of AFB is burning the symptomatic colonies (Alippi, 2014; Genersch, 2010; Matović et al., 2023). AFB issue is intensified in countries, such as Estonia, with no specific action plan for AFB treatment, or it is incomplete owing to inadequate legislation, or there is no compensation for burnt colonies (Mänd et al., 2022). This enables the persistence and spread of *P. larvae* spores and remains a serious threat to the beekeeping sector. Another problematic issue is the underreporting of AFB by beekeepers. For example, in 2010–2015, no or very few annual AFB outbreaks were reported in the Abruzzo region of Italy. However, *P. larvae* levels in the sampled honey from that region showed a prevalence of at least 22.7% (Ricchiuti et al., 2019). We are not able to prove whether the beekeepers, who participated in this study, did have any clinical outbreak of AFB or not We suspect that the reviewer may have confused vertical lines with horizontal lines. We added horizontal lines to Table 2, and we believe that vertical lines are not necessary if the reviewer did not make a mistake.

In the current study, the hive density was positively correlated with *P. larvae* load in bee colonies. Indeed, *P. larvae* spreads horizontally from bee to bee (Fries & Camazine, 2001), so it is favoured in regions where the beehive density is high. *Paenibacillus larvae* spore transmission may be especially high if robbing between honey bee colonies occurs and the inter-colony distance is within a radius of 1 km (Lindström et al., 2008). Our results, however, indicated that the number of *P. larvae* spores was positively correlated even with the number of neighbouring apiaries and bee colonies present within a 7 km radius. Higher honey bee colony densities may be a major risk factor for other diseases or parasites like European foulbrood (EFB – caused by *Melissococcus plutonius*) and *Varroa destructor* (Büren et al., 2019; Frey & Rosenkranz, 2014, Piot et al., 2022), for instance. The horizontal spread of the parasites can be enhanced by the secondary distribution made by other flower visitors (Karise et al., 2016).

The present study also revealed that climatic conditions can favour or hinder the presence of *P. larvae* spore load in beehives. According to Tóth et al. (2016), Estonia is divided into two climatic zones: boreal to subboreal and northern subcontinental. From north to south, the climatic differences are relatively small; however, from east to west, the impact by continental air masses bringing harsher weather is replaced by a milder weather impacted by the maritime air masses from the Atlantic Ocean. We observed a significantly higher number of *P. larvae* spores over western areas, pointing to the potential impact of local weather conditions. The spread of varroosis and nosemosis have also been related to the variable climatic conditions (Lotfi & Shahryar, 2011; Naudi et al., 2021). Other stress factors like agricultural intensity (Hart et al., 2022), pesticides (Raimets et al., 2018, 2021), but also coeffect of different factors (Pent et al., 2023) decrease the health status of the honey bees and make them even more susceptible to any new coming threats including the AFB (De Rycke et al., 2002; López et al., 2017). Therefore, beekeepers' ability to keep honey bees in good health could be improved by selecting appropriate apiary locations in addition to managing the diverse stress factors to lower AFB risk. Nationally, the honey bee health situation could be implemented using appropriate education plans (De Graaf et al., 2016; Mänd et al., 2022) and learning support (Guiné et al., 2021, 2023).

It seems that changes in the spread of *P. larvae* among honey bees are occurring. The breeding of high-quality queen bees over the last decade has allowed the presence of spores in colonies without the clinical symptoms of AFB (Matović et al., 2023; Spivak & Reuter, 2001). Challenges, like Varroa mites, have forced beekeepers to adopt professional queen bee breeding, which might also impact the honey bee susceptibility to AFB. This includes selecting queens with better hygienic behaviours for breeding. Bees with high hygienic behaviour have reduced AFB susceptibility (Spivak & Reuter, 2001). Nurse bees removing diseased larvae early show decreased clinical symptoms, preventing AFB onset (Matović et al., 2023). Improved hygiene also decreases other diseases, aiding resilience to infections. Hygienic beekeeping practices and selecting queens from hygienic lines are crucial for colony health (Leclercq et al., 2017).

In the future, preventive or curative measures may be possible through the vaccination of queen bees (Dickel et al., 2022) or by using targeted veterinary products based on propolis, honey, and non–hive sources (Borba & Spivak, 2017; Wilson et al., 2017; Ye et al., 2023). For now, knowing the number of *P. larvae* spores in sampled honey bee colonies is

valuable information, assisting in preventing disease outbreaks through hygienic measures. Forsgren and Laugen (2014) showed that the analysis of pooled samples of bees and debris was the most effective way for *P. larvae* detection (Forsgren & Laugen, 2014). Single hive sampling instead of pooling certainly helps to uncover the potential infection sources within apiaries. For routine regional screening, however, pooling the samples per apiary would be cost-effective way with no dependence on apiary size.

Conclusions

The current study provides a snapshot of *P. larvae* spore distribution in Estonian apiaries identifying the high apiary density and climatic conditions as factors increasing the risk of outbreaks of AFB. Therefore, veterinary surveillance should be intensified in areas with high apiary densities and specific climatic zones. The absence of AFB signs does not reflect the actual *P. larvae* spore loads in apiaries and easy sampling of hive debris followed by rapid qPCR analysis facilitates the identification of infection hotspots for appropriate management. In addition, future research should establish a more reliable relationship between spore load in different matrices and the clinical symptoms of AFB to improve risk estimation based on *P. larvae* quantification. Quantification of *P. larvae* should be intensified in regions with mild climatic conditions that might favour infection especially in areas with high colony densities.

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Translocation of Tebuconazole between Bee Matrices and Its Potential Threat on Honey Bee (*Apis mellifera* Linnaeus) Queens

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Simple Summary: Numerous pesticide residues have been found in bee products. It is unclear whether and to what degree pesticides migrate between different bee matrices. Even though the use of many common insecticides is strictly regulated, fungicide residues are still ubiquitous in bee matrices and data regarding this problem are still insufficient. The aim of this work was to determine the migration of fungicide tebuconazole between bee matrices and to assess its potential risk to honey bee queens. We found that tebuconazole mixed into wax has the potential to migrate into royal jelly (RJ), but no residues were found in honey bee queen larvae and newly emerged queens. The residues of tebuconazole found in queen cell cups and RJ decreased over time and probably posed no direct lethal threat to queens. Nevertheless, sub-lethal effects of tebuconazole on honey bee queens might occur even at low concentrations.

Abstract: Various pesticide residues can be found in different bee colony components. The queen larvae of honey bee (*Apis mellifera* L.) receive non-contaminated food from nurse bees. However, there is little knowledge about how pesticide residues affect developing bees. Additionally, little is known about the migration of lipophilic pesticides between bee matrices. While wax, royal jelly (RJ), and bee larvae are chemically distinct, they all contain lipids and we expected the lipophilic fungicide tebuconazole to be absorbed by different contacting materials. Our aim was to analyze the translocation of tebuconazole residues from queen cell wax to RJ, queen larvae, and newly emerged queens and to evaluate its potential risk to queens. We demonstrated the potential for the migration of tebuconazole from wax to RJ, with a strong dilution effect from the original contamination source. No residues were detected in queen bee larvae and newly emerged queens, indicating that the migration of tebuconazole probably did not directly endanger the queen bee, but there was some risk that tebuconazole might still affect the homeostasis of developing bees.

Keywords: translocation of pesticides; wax; hazard quotient; tebuconazole; honey bee queen

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1. Introduction

Pesticides are known to be amplify all stress elements related to honey bee colony losses [1]. Various pesticides accumulate in different bee products [2,3] and often beeswax is the most contaminated bee product [3–5]. Most of the conducted studies have been focused on insecticide effects on bees, while fungicides have gained little attention. Nevertheless, it has been shown that at least azole type fungicides can potentiate the toxicity of insecticides [6–8].

Azole fungicides are among the highest volume fungicides used against fungal diseases of agricultural crops [9]. Among other azole fungicides, tebuconazole is very commonly used and its residues have been found in bee matrices [2,10,11]. Tebuconazole has synergistic negative effects on honey bees together with insecticides [10]. Additionally,

it can separately affect microbial communities in different environments, including those in insect guts [11,12].

The large majority of bee toxicology studies have been focused on the impact of pesticide residues on worker bees [13–15], while queens have received little attention. The main task of a mated queen is to sustain colony development and survival via laying eggs [16]. During their lifetime, queens are fed pure royal jelly (RJ) secreted by nurse bees [17] that feed on nectar, pollen, and beebread, which can be contaminated by various pesticides [3,18–20]. Contaminated pollen and nectar can lead to contamination of beeswax, which absorbs lipophilic compounds well [3,5,21,22]. This may pose long-term risk to the viability of a beehive. Although the queen bee is protected from xenobiotic compounds due to feeding solely on RJ, it spends most of its life on or in wax combs. It has been shown that honey bee larvae can suffer morphological changes when grown on contaminated wax [23]. The same changes may occur in queen larvae, because the ambient conditions are the same during queen development.

Long-term contact with pesticide residues exposes bees to a certain risk. One way to quantify the potential risk is to calculate a hazard quotient (HQ) [21]. HQ is calculated by dividing the concentration of contaminant in wax with the LD_{50} value (the concentration that would kill 50% of the test group individuals) of the substance. HQ simply evaluates the risk of individual contaminants to bees. However, depending on their specific tasks in colony, the individual honey bees are in contact with different materials [3]. In the case of multiple contaminants, the calculation of HQ still helps to understand the contribution of individual contaminants to the overall risk.

The contaminating molecules are able to migrate between materials inside the hive [22]. The migration ability depends on the chemical nature of the contaminant, on the chemical and physical characteristics of the material.

The information of pesticide migration among bee products is still scarce. For instance, Böhme et al. showed that RJ is not contaminated [24], despite the contaminated pollen they were fed with. Similar results were shown by Johnson and Percel, where nurse bees that were fed contaminated pollen secreted pure RJ [25]. However, contamination of RJ can occur via exposure to wax comb cells.

The purpose of this study was to test whether tebuconazole residues mixed into honey bee wax migrate from one bee product to another (RJ; queen bee larvae; newly emerged queens). The aim thereafter was to evaluate the potential hazard to queens.

2. Materials and Methods

2.1. Honey Bees Used

The experiments were conducted in summer 2020 at a single apiary (OÜ R-honey) located in the Eastern region of Estonia. Queens (*Apis mellifera ligustica*) were bred in the experiment from one-day old larvae originating from a single queen. Queenright normal-sized honey bee colony (50 Langstroth frames) was used as a cell builder. An egg laying queen was allocated to the first Langstroth box on the hive bottom and separated with queen excluder. In addition, an extra flight entrance was installed above the queen excluder to encourage building up the queen cells by nurse bees. Honey supers were allocated on the extra flight entrance and the grafted queen cells with open brood frames were all inserted into the top box.

2.2. Exposure to Tebuconazole

Wax obtained from a local organic beekeeping operation was used for making queen cell cups. The active ingredient tebuconazole (purity 99.3%) was purchased from Sigma Aldrich. Tebuconazole was dissolved in acetone and incorporated into molten wax. The tebuconazole concentration mixed into wax was 412 µg kg^{-1}. Field-realistic pesticide concentration was selected based on the analysis of Estonian bee products by Raimets et al. [26]. In addition, tebuconazole has been found in honey bee wax in other studies [2,3,6].

Immediately after the mixing of tebuconazole into molten wax, queen cell cups were prepared using special wooden dowel to shape the cup. Separate dowels were used for the control and test group cups. It is a common procedure in beekeeping to make queen cell cups from molten wax [26]. One-day-old honey bee worker larvae were grafted into the newly made cups. A frame with the grafted cups (control cups (n = 20)) and tebuconazole spiked cups (n = 20) was inserted into queenright cell builder colony. After 24 h, the queen cell acceptance by nurse bees was controlled visually.

The closest conventionally managed agricultural crops were located 5.4 km away. The experiment was conducted in July, which is the main honey flow period in Estonia. At that time, there is abundance of plants blooming in the wild. Considering the distance between the apiary and conventional farming fields, it is very unlikely that the bees visited conventional farming fields and were exposed to tebuconazole from outside environment.

2.3. Pesticide Residues in Bee Matrices

In order to investigate whether tebuconazole can migrate from one bee product to another, we collected queen cell cups, RJ, accepted queen larvae, and newly emerged queens. RJ and larvae were collected from cups 3 days after the acceptance. RJ was collected using micropipette and larvae were taken from the cells using a special spatula. Adult queens and built-up queen cell cups were taken for pesticide residue analysis on the day of their emergence. All the samples were put into freezer (−20 °C) immediately after the collection. While large volume experiments with honey bee queens are difficult, we could not split the individually treated cells into several groups to obtain the required minimal sample mass (2 g) for pesticide residue analyses. Therefore, the samples were pooled according to the types of bee matrices. The pooled samples were sent to a laboratory (Institute of Food Safety, Animal Health and Environment "BIOR", Riga, Latvia) for pesticide analysis.

2.4. Pesticide Residue Analyses from Bee Matrices

Tebuconazole residues from honey bee matrices were analyzed at the Institute of Food Safety, Animal Health and Environment "BIOR". The UHPLC-MS/MS assay was performed using an Ultimate 3000 high performance liquid chromatograph (Thermo Scientific, MA, USA) coupled to TSQ Quantiva tandem mass spectrometer (Thermo Scientific, MA, USA).

2.5. Chemicals and Materials Used in Pesticide Residue Analyses

Pesticide reference standards were supplied by Dr. Ehrenstorfer (Germany). Methanol and HPLC grade acetonitrile were acquired from Merck Millipore (Darmstadt, Germany). Ammonium formate (99%), acetic acid (glacial, >99.85%), and ACS grade formic acid (>96.0%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure deionized water was prepared by using a Millipore Milli-Q™ system (Billerica, MA, USA). The following substances were purchased from Phenomenex (Torrance, CA, USA): buffer salt mixture (1 g trisodium citrate dihydrate, 1 g sodium chloride, 0.5 g disodium hydrogen citrate sesquihydrate, and 4 g of anhydrous magnesium sulphate) and a mixture of dSPE (900 mg anhydrous magnesium sulphate, 150 mg PSA, and 150 mg C18E). Stock solutions (approximately 1000 mg L^{-1}) were made by weighing 10 mg of standard in a 10 mL graduated flask and then dissolving in acetonitrile. While preparing the standard solutions of final concentration, the purity of standard was taken into account. In order to prepare a working standard solution with a concentration of 0.01 mg L^{-1}, the appropriate volume of stock solution was diluted with acetonitrile. The prepared solutions were stored at 20 °C.

2.5.1. Sample Preparation

The sample (2.0 ± 0.1 g) was weighed into a 50 mL centrifuge tube. The standard solutions were added at the appropriate spiking level in order to prepare calibration and quality control samples. Acetonitrile (10 mL) and deionized water (10 mL) were both added and the tubes were shaken vigorously by hand for 1 min. Then, a mixture of trisodium

citrate dihydrate (1 g), sodium chloride (1 g), disodium hydrogen citrate sesquihydrate (0.5 g), and anhydrous magnesium sulphate (4 g) was added, the tubes were closed, shaken for 10 min, and centrifuged for 10 min at 3500 rpm.

The supernatant was transferred into a 15 mL PP centrifuge tube and frozen out at −70 °C for 30 min using a Heto Ultra freeze (Thermo Fisher Scientific, MA, USA), followed by centrifugation of the resulting organic sample fraction for 10 min at 3500 rpm. For further clean-up procedure, 6 mL of the extract was transferred into 15 mL PP tubes, each containing anhydrous magnesium sulphate (900 mg), PSA (150 mg), and C_{18} sorbent (150 mg). The tubes were shaken vigorously by hand for 30 s and then centrifuged for 10 min at 3500 rpm. A 250 µL aliquot of the purified extract was mixed with 500 µL of the mobile phase A, consisting of 5 mM ammonium formate and 0.1% formic in water and filtered through a 0.22 µm PVDF membrane centrifuge filter. An aliquot of the extract was transferred to an autosampler vial for UHPLC-MS/MS analysis.

2.5.2. UHPLC-MS/MS Analysis

For pesticide analyses in samples, an UltiMate 3000 high performance liquid chromatograph coupled to a TSQ Quantiva tandem mass spectrometer equipped with an electrospray ionization source was applied. The parameters of the ion source were the following: vaporizer temperature was adjusted at 450 °C and ion transfer capillary at 320 °C, ion spray voltage 3.5 kV (positive mode), sheath gas 45 arbitrary units (arb), auxiliary gas 25 arb, and sweep gas 4 arb. The analysis was performed by multiple reaction monitoring (MRM) in the positive ionization mode. Table 1 lists the analyte-dependent parameters: MRM transitions and collision energies (CE).

Table 1. Instrumental parameters of the applied method.

Pesticide	Molecular Ion, Da	Daughter Ion, Da	Collision Energy, eV
Tebuconazole	308	70	21
	308	125	34

Chromatographic separation was performed on a Kinetex C18 analytical column (50 × 3.0 mm, 1.7 mm) from Phenomenex. The mobile phase A consisting of 5 mM ammonium formate and 0.1% formic in water and acetonitrile (mobile phase B) were delivered at the flow rate of 0.4 mL min^{-1}. A gradient program was used: 20% of mobile phase B was used from 0 to 1.0 min, 20% (B) to 90% (B) from 1.0 to 10.0 min, maintained at 90% (B) for 1 min, then decreased back to 20% (B) at 11.0 min and finally the column was re-equilibrated with 20% (B) from 11.0 to 15.0 min. A 10 µL aliquot of the extract was injected. The column and autosampler were maintained at 30 °C and 10 °C, respectively.

2.6. Statistical Analysis

For calculating the hazard quotient (HQ), the wax toxicity calculation tool (Bee Tox Wax) was used [21]. The aim of HQ was to evaluate the potential exposure to a pesticide and to set a level at which no adverse effect is expected. When using the "Bee Tox Wax" tool, the median lethal dose (LD_{50}) is a quantitative indicator of the pesticide toxicity. In this case, HQ values under 250 were shown to be slightly toxic to bees and the wax can be re-used.

3. Results

3.1. The Residues Detected

UHPLC-MS/MS assay showed that tebuconazole was present in certain bee matrices (Table 2). Tebuconazole residues were found in the entire queen cell wax (0.19 mg kg^{-1}), indicating that this substance was incorporated into wax during the experiment. The residues were also found in RJ samples but already at lower concentrations. The tebuconazole concentration found in RJ was 0.08 mg kg^{-1} (0.08 µg per bee), which was 2.4 times

lower than found in wax and probably should not pose a risk to the survival of honey bee queens. Despite the fact that tebuconazole was present in queen cell cups and RJ, its residues were not found in honey bee queen larvae and newly emerged queens. It could be assumed that queen larvae and adult queens metabolised tebuconazole and thus no residues were found. Nevertheless, the results show the potential for tebuconazole migration from wax to RJ.

Table 2. Tebuconazole residues detected in bee matrices after spiking queen cell cup wax with tebuconazole at the concentration of 0.412 mg kg^{-1}. Additionally, the HQ values of tebuconazole residue in wax are shown. According to the "Bee Tox Wax" tool data, a HQ value under 250 indicates low wax contamination rate [21].

Bee Matrix.	Control (Tebuco-Nazole Found (mg kg^{-1})	Tebuco-Nazole Found (mg kg^{-1})	% Left from Spiking Concentra-Tion	Tebucona-Zole Contact LD_{50} to Honey Bees 48 h (mg kg^{-1})	Dose (µg) per Bee Found	HQ Value
Queen cells	<0.01	0.19 ± 0.09	46.1	0.2	0.19	1
Royal jelly	<0.01	0.08 ± 0.04	19.4	0.2	0.08	
Larvae	<0.01	<0.01	0	0.2	0	
Queens	<0.01	<0.01	0	0.2	0	

3.2. Hazard Quotient

According to the findings of tebuconazole residues in wax, the Hazard Quotient (HQ) was used to determine its potential toxicity level to honey bee queens. The HQ value of initial concentration mixed into wax was 1.

The tebuconazole concentration found in queen cells was 0.19 mg kg^{-1} (0.19 µg per bee), which means that it should not cause direct mortality of bees, but this sub-lethal concentration may have significant negative effect on queen homeostasis. In the case of tebuconazole, 200 µg per bee has been shown to be the acute lethal contact dose after 48 h. The HQ value in our case was 1, which means that the wax was slightly contaminated and it can be used in beekeeping and recycling. According to the Bee Tox Wax tool, any HQ value under 250 is considered to be low-polluted and the wax could be used and recycled in beekeeping operations [21]. At the same time, it should be taken into account that the risk for bees will increase with increasing number and levels of different contaminants.

4. Discussion

This study has demonstrated the migration of tebuconazole from wax to RJ. The only source of tebuconazole was the addition of this xenobiotic compound to molten wax. However, further migration from RJ to queen larvae or newly emerged queens did not occur. Despite the high initial concentration, the content of tebuconazole detected from the entire queen cell wax was lower.

Lipophilic pesticides like tebuconazole can accumulate in wax [3,27] and, due to continuous agricultural application, the amount of residues in bee matrices can be expected to increase with time. Little is known about the effects of certain pesticides on queen bee performance. In addition, various studies show that different pesticides can cause synergistic effects in bees. Raimets et al. showed that azole fungicide imazalil had a synergistic effect with three insecticides on the mortality of bumble bee (*Bombus terrestris*) [6]. Besides, Vandame and Belzunces showed that insecticide deltamethrin had a synergistic effect with azole fungicides, causing changes in bee thermoregulation [28]. Azole fungicides inhibit cytochrome P450 detoxification system in bees [7] and thus the bees are more vulnerable to other pesticides.

The octanol-water partition coefficient of tebuconazole (Log P_{OW}) has been shown to be 3.7 [29], which means that this compound has lipophilic properties and can concentrate in lipophilic bee matrices. In addition, tebuconazole is quite stable at elevated temperatures and its melting point is 102.4 °C [29], while beeswax melting point is about 63–65 °C [30].

This also confirms that tebuconazole incorporation into molten wax is possible without degradation.

It is vital to understand whether pesticide residues from wax can be taken up by developing honey bees. Medici et al. showed that the presence of insecticides in wax negatively affected honey bee brood survival [31]. There have been only a few studies focused on the migration of pesticides from wax to bees, especially to queen bees. A study conducted by Böhme et al. showed that some pesticide residues (with different Log *p* values) can be found in RJ, though in apparently negligible concentrations (the highest concentration found was 0.016% of the original concentration fed to the nurse bees) [24]. In another experimental study, hives were treated with known amounts of tau-fluvalinate via contaminated plywood inserts, and no residues were detected in RJ [32]. On the contrary, our study revealed that fungicide tebuconazole was translocated from polluted wax to RJ. Nevertheless, tebuconazole concentration found in RJ was 2.4 times lower than in wax. Another study showed that nurse bees fed on contaminated pollen and nectar produced uncontaminated RJ for honey bee queens [33]. Our study revealed that RJ can be contaminated via exposure to residues in wax. Our results are in accordance with Milone and Tarpy who showed that different pesticides mixed into queen cell cups successfully migrated to RJ [34].

Larval feeding pattern performed by nurse bees is different among honey bee castes and sexes (workers, drones, queens). In the case of queen larvae, feeding was performed by nurse bees within relatively short visits and the content of RJ remained almost constant, while worker larvae received longer feeding periods after 48 h [35]. In addition, Dietz and Lambremont showed that honey bee queen larvae consume 13% more food than worker larvae within first the 3 days of larval development [36]. Thus, the higher food consumption by queen bee larvae may simultaneously increase the amount of ingested pesticide residues in the case if RJ is contaminated.

Tebuconazole concentrations found in bee matrices are related to the lipophilicity of this compound, which tends to accumulate in nonpolar media. This tendency was demonstrated in our experiments where the highest concentration was revealed in wax (very non-polar matrix), lower concentration was detected in RJ (up to 6% of fats), and the lowest content in larvae (mostly water-protein sample). Due to the chemical and physical properties of tebuconazole, it preferentially remained in the samples with the highest lipid content. Additionally, tebuconazole has been shown to be persistent in soil (aerobic metabolism $T_{1/2}$ in soils is 796 days) [37]. Even under hydrolytic conditions, it was stable for >28 days. Therefore, the most probable reason for the occurrence of tebuconazole in certain bee products is related to its tendency to stay in a lipophilic environment.

It is noteworthy that there was no conventional farming within 5.4 km radius in our study, which excludes the possibility of bee exposure from agricultural use of tebuconazole. Morales et al. showed that pesticides used on the fields, as well as in apiculture, can move from beeswax to honey bee brood [38]. Interestingly, our results did not indicate tebuconazole residues in the sampled honey bee queen larvae and newly emerged queens. We saw rapid dilution of tebuconazole through different matrices. Compared to the cell cup wax, the whole queen cells contained less than one half the concentration of tebuconazole. Due to the dilution with newly added wax, the initial concentration mixed into queen cell cups had decreased 2.2 times. In addition, the concentration of tebuconazole decreased 2.4 times when comparing RJ to wax. However, if the apiary had been located next to conventional farming lands, the amount of tebuconazole residues in bee matrices could be considerably higher due to the agricultural use of tebuconazole.

Besides chemical decomposition of pesticide molecules over time, the individual bee organisms are also capable of detoxification [39]. In addition, Berenbaum and Johnson have proposed that detoxification of xenobiotic compounds among eusocial honey bees may be complemented by a "social detoxification system", which includes colony food processing via microbial fermentation, dilution by pollen mixing, and worker discrimination [40]. Without detoxification, the effects of pesticides on honey bee health could be more severe.

Some studies have shown that pesticides may have effects on the longevity, olfactory functions, and water consumption by bees during long-term exposure [41,42]. Nevertheless, in our case, the concentration of tebuconazole after 3 days of exposure was not observed, probably due to the inability of this lipophilic contaminant to migrate from wax to biological tissues of larvae and queen bees.

Tebuconazole concentrations found in bee products in our study probably do not possess any direct lethal effect to developing queens. Still, pesticides even at low concentrations have been shown to cause sub-lethal effects in bees [43]. Fungicide tebuconazole is considered to have low toxicity to bees [44]. Despite the fact that tebuconazole probably does not kill bees directly, it might cause changes in bee homeostasis. As it is known, there are eight different dominant bacterial species in honey bee gut and they tend to exhibit strain diversity according to differences in tolerance of pesticide exposure and metabolic capability [45]. Tebuconazole at high concentrations (5, 50, and 500 mg kg^{-1}) has been shown to decrease soil microbial biomass and activity but, interestingly, no clear effect of different concentrations was found [11]. In addition, tebuconazole reduced gut fungal diversity in brown planthopper (*Nilaparvata lugens* Stål). Interestingly, a study conducted by Powell et al. showed that newly emerged honey bees are lacking gut bacteria and their gut is colonized 4–6 days after the emergence via contacts with other workers [46]. Even though tebuconazole does not spread from RJ to bee larvae, a possibility of tebuconazole exposure inside the hive (contaminated pollen, beebread, etc.) cannot be completely excluded. Changes in bee gut microbiota are shown to increase the susceptibility to diseases [47] and bee mortality may be increased due to different sub-lethal effects [45].

5. Conclusions

The current pilot study demonstrated the migration of a single pesticide from one bee product to another. Further studies are needed to investigate the potential for the migration of multiple pesticides among bee products, with the goal to identify and quantify the impact on bees associated with possible synergistic and sub-lethal effects.

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IV

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Effect of Nosema apis and N. ceranae on honey bee Apis mellifera queen development

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Nosema apis and N. ceranae are agents causing the disease called nosemosis in honey bee workers and queens. Few is known about the impacts of it on honey bee development. The royal jelly in queen cells was infected with *Nosema* spores to see whether and how it affects the development of honey bee queens. Seven groups of grafted honey bee larvae were established, and treated as follows: high and low concentrations of *N. ceranae* and *N. apis*, mixes of both species in both concentrations, and untreated control. After allowing nurse bees to fill the queen cells with royal jelly, an injection of 50 000 spores or 10 000 spores was added into the royal jelly. We found that only *N. apis* decreased the hatching rate of honey bee queens both in single and mixed treatment at high dosages, but we did not detect any morphological deviations in unhatched pupae.

Key words: honey bee *Apis mellifera*, *Nosema spp*., honey bee queen breeding, queen quality

Introduction

Honey bees (*Apis mellifera* L.) are suffering from high colony mortality rates in Europe [11]. There are several reasons for that and pathogens are considered as most important causes [3]. One of the most damaging diseases, nosemosis is caused by microsporids *N. apis Zander* [7] and *N. ceranae Fries* [8], which can also co-exist in the same colony.

Nosemosis has been under scientific interest for a long time whereas most of the work has been conducted on honey bee workers. The queens, however, can also be susceptible to nosemosis. *Nosema*-infected queens have been seen to produce a higher amount of queen mandibular pheromone, which refers to the poorer quality of the queen [1]. Nosemosis is often linked to changing vitellogenin titers in worker and

queen bees, although, the effects can be different [2]. Changes in vitellogenin amount affect the queen's hormonal balance, oxidative stress, and longevity [5]. Still, only a few studies consider the effects of nosemosis on honey bee queen development, and to our knowledge, there are none considering the developmental success due to manipulative infection. While nosemosis is detected not only the intestine but also hypopharyngeal glands of honey bees, the royal jelly produced by nurse bees can be infected with Nosema spp. spores [4].

The aim of this study was to explore i) whether *Nosema spp*. spores can be transferred from royal jelly to emerged queens and ii) whether the infection causes any morphological deviations.

Materials and Methods

The study was conducted in the summer of 2021. New queens (*A. mellifera ligustica*) were bred from one-day-old larvae originating from a single queen. Colonies used for nursing (royal jelly producing, cell building) were repeatedly checked for three weeks prior to the experiment to ensure that there was no infection present.

To obtain fresh *N. apis* and *N. ceranae* spores, infected honey bee colonies were used. From those, infected worker bees were collected and the spores were separated by homogenization. The *Nosema* species were identified by a multiplex PCR (M-PCR) assay [10]. Once the species was known, a centrifugation protocol [9] was used to purify the spores, which enabled about 85% purity. In order to separate the last precipitate, the resulting suspension was cleaned with a 10-micron filter. A flow cytometer (BD Accuri C6) was used to determine the number of spores [10].

The queen cell cups were made of organic bees wax (purchased from a local producer) to reduce the risk of pesticide contamination, which could affect the outcome of the experiment. The one-day-old larvae were grafted into the cups. A total of seven treatment groups were created with ten larvae for each: *N. apis* low (10000 spores), *N. apis* high (50.000 spores); *N. ceranae* low, *N. ceranae* high; Mix low, Mix high; Control.

After grafting the cell builder colony was checked regularly until the cups were sealed by worker bees. If a cup was sealed, an injection of 2 µL of suspension was added through the wax walls of the cup into the royal jelly (ddH_2O and nosemosis agents). Larvae were located, directing (a strong) light through the wax cell, and the suspension was injected underneath the larvae. On the tenth day after the grafting, queen cups were isolated (using Nicot's queen cages) and taken into an incubator (at 35°C and 65% humidity) until hatching.

Results and Discussion

The hatching rate of honey bee queens was affected by the treatment (**Fig. 1**). Interestingly, with *N. apis* high spore load treatment, the hatching rate was zero, while it was not affected by the same spore load of *N. ceranae*.. The dissection of sealed queen cells showed variable developmental deviations in this group (*N. apis* high), most of these queens had ended their development at the larval stage and didn't reach the metamorphosis. Only a couple of individuals started the pupation but stopped suddenly before finishing it as shown in **Fig. 2**. Similarly, a low hatching rate was observed in the mixed high concentration group. We suggest that *N. apis* caused this.

The hatching rate of other treatment groups was relatively normal. Usually, beekeepers aim to achieve a hatching rate of over 80% when breeding the queens. *N. apis* has been considered problematic for honey bees during the early season [7], but our study reveals yet another threat – honey bee breeding success can be seriously harmed when the nursing colonies are infected and not checked for this disease. There are no clear symptoms of nosemosis and only laboratory analyses of bee samples allow a correct diagnosis of this disease.

Some papers indicate that nosemosis agents are able to transfer through the metamorphosis process in worker bees [2, 6, 12], although, we could not confirm this in queen bees – we did not detect any infection from the adult newborn queens.

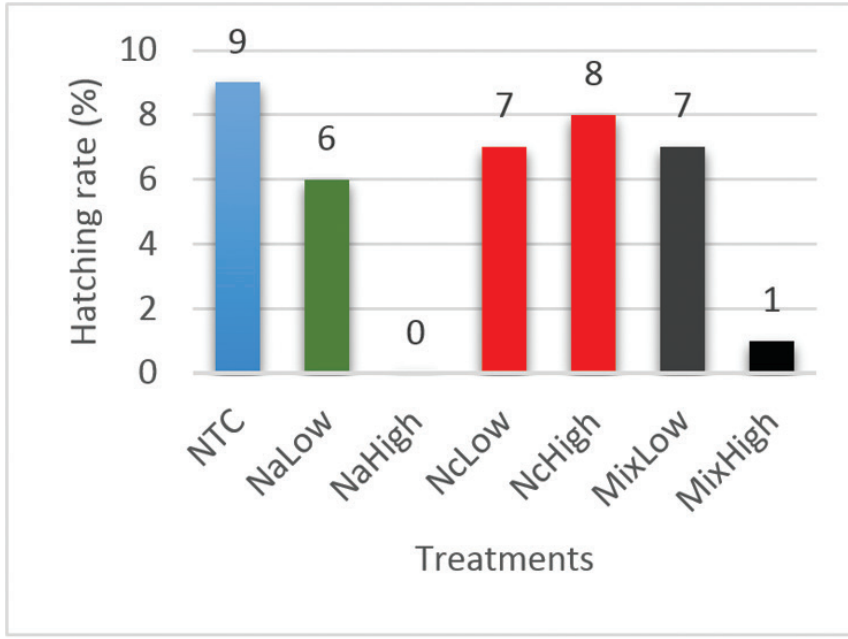

Fig. 1. The hatching rate of the treatment groups. NTC-no treatment control. Na –*N. apis*, Nc –*N. ceranae*, Mix – Na + Nc, low – 10 000 spores, high – 50 000 spores injected intoroyal jelly in queen cells.

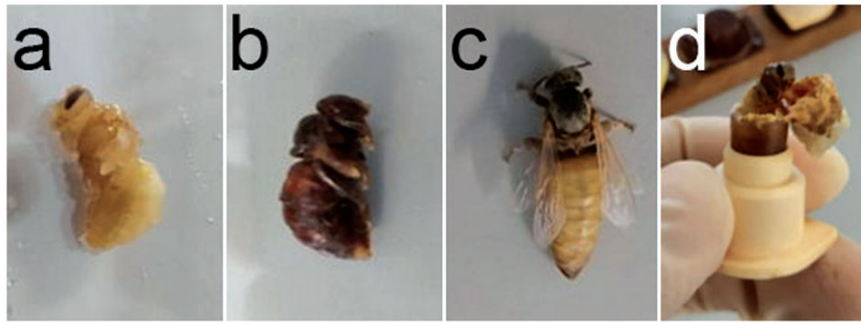

Fig. 2. High doses of *N. apis* both as pure treatment (a and b) or in mix with *N. ceranae* (c and d) caused disruption of metamorphosis at different developmental timepoints (Photos by S. Naudi)

Conclusions

Our study showed that royal jelly contamination with high concentrations of *N. apis* can cause honey bee queen mortality during breeding. This first study on *Nosema* affecting queen development through contamination of royal jelly is indicating the need for more targeted research on this topic and is really important for the honey bee queen breeding system.

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12. Traver, B. E., R. D. Fell. Low natural levels of nosema ceranae in apis mellifera queens. – *Journal of Invertebrate Pathology*, SIP Symposium on Resistance to BtCrops., **110 (3)**, 2012, 408–410.

CURRICULUM VITAE

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Academic Degree:

2017 Master's Degree in Nature Tourism, Estonian University of Life Sciences. Thesis: "Pesticides tebuconazole and tau-fluvalinate impact on honeybee queen development" Supervisors: Marika Mänd, Reet Karise, Risto Raimets

Career:

2018-2021 Estonian University of Life Sciences, Institute of Agricultural and Environmental Sciences, Chair of Plant Health, Junior Researcher (1.00)

2021-2025 Estonian University of Life Sciences, Institute of Agricultural and Environmental Sciences, Chair of Plant Health, Specialist (1.00)

Education:

2017-2025 Doctoral studies in Agriculture, Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences

2015-2017 Estonian University of Life Sciences, Institute of Agricultural and Environmental Sciences, Master's Degree in Nature Tourism

2012-2015 Estonian University of Life Sciences, Institute of Agricultural and Environmental Sciences, Bachelor's Degree in Nature Tourism

2009-2012 Tartu Forseliuse Gymnasium, Secondary Education

2000-2009 Tartu Forseliuse Gymnasium, Basic Education

Honours and Awards

2023	Kristjan Jaagu Scholarship for participation in the Estonian-Bulgarian cooperation project in Bulgaria
2022	Dora Plus short-term mobility scholarship for participation in the Estonian-Bulgarian cooperation project in Bulgaria
2022	Dora Plus short-term mobility scholarship for participation in the NBBC conference with an oral presentation in Denmark
2019	Dora Plus short-term mobility scholarship to participate in Apimondia 2019, Canada, with a poster presentation
2018	ASTRA "Value Chain Bioeconomy" Scholarship to participate in the international conference "EurBee 8" in Belgium
2018	Dora Plus short-term mobility scholarship to participate in the Nordic-Baltic Apicultural Symposium in Riga
2017	Dora Plus short-term mobility scholarship to participate in the course "Sustainable Insect Management and Disease Control Based on RNAi Technology" at the University of Helsinki

Administrative Work

2023-…	Coordinator of the Hive Scale Network, Support for Intervention in the Estonian Beekeeping Sector 2023–2025
2023-…	Member of the Estonian Beekeeping Assembly
2022-…	Member of the Board, Estonian Professional Beekeepers Association
2019-…	Member, Estonian Professional Beekeepers Association
2017-…	Board Member, Simmu Mesi OÜ
2019-2023	Coordinator, Horizon2020 Project "PoshBee"
2020-2022	Project Manager, L210003PKTE "Hive Scale Data Analysis (1.06.2020–30.04.2022)"
2019-2022	Sub-action Manager, National Honey Program 2020
2022-…	Hivescales management, analysis

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Teaduskraad:

2017 Magistrikraad Loodusturismi erialal Eesti Maaülikoolis. Lõputöö teema: "Pestitsiidide tebukonasooli ja tau-fluvalinaadi *mõju mesilasemade arengule"* Juhendajad: Marika Mänd, Reet Karise, Risto Raimets

Teenistuskäik:

2018-2021 Eesti Maaülikool, Põllumajandus- ja keskkonnainstituut, Taimetervise õppetool, nooremteadur (1,00)

2021-2025 Eesti Maaülikool, Põllumajandus- ja keskkonnainstituut, Taimetervise õppetool, spetsialist (1,00)

Haridustee:

2017-2025 Eesti Maaülikool, Põllumajandus ja keskkonnainstituut, doktoriõpe

2015-2017 Eesti Maaülikool, Põllumajandus- ja keskkonnainstituut, Loodusturismi eriala, magistriõpe

2012-2015 Eesti Maaülikool, Põllumajandus- ja keskkonnainstituut, Loodusturismi eriala, bakalaureuseõpe

2009-2012 Tartu Forseliuse Gümnaasium, Keskharidus. Täiendkutse: rekreatsiooni eriala Tartu Kutsehariduskeskus

2000-2009 Tartu Forseliuse Gümnaasium, Põhiharidus

Teaduspreemiad ja tunnustused

2023 Kristjan Jaagu stipendium osalemiseks Eesti-Bulgaaria koostööprojektis Bulgaarias

2022 Dora Pluss lühiajalise õpirände stipendium osalemiseks Eesti-Bulgaaria koostööprojektis Bulgaarias

2022 Dora Pluss lühiajalise õpirände stipendium osalemiseks NBBC konverentsil suulise ettekandega Taanis

2019 Dora Pluss lühiajalise õpirände stipendium osalemiseks ühel maailma suurimal mesinduskonverentsil (Apimondia 2019, Kanada) posterettekandega

2018 ASTRA "Väärtusahela põhine biomajandus" stipendium osalemiseks rahvusvahelisel konverentsil "Eurbee8" Belgias, Gentis, posterettekandega

2018 Dora Pluss lühiajalise õpirände stipendium osalemiseks Põhja- ja Baltimaade iga-aastasel sümpoosiumil Riias, suulise ettekandega Lätis

2017 Dora Pluss lühiajalise õpirände stipendium osalemiseks Helsingi Ülikoolis kursusel "Sustainable Insect Management and Disease Control Based on RNAi Technology"

Administratiivtöö

2023-… Eesti Mesindussektorisse sekkumise toetus 2023–2025, Tarukaalude võrgustiku koordinaator

2023-… Eesti Mesinduskogu liige

2022-… Eesti Kutseliste Mesinike Ühing MTÜ juhatuse liige

2019-… Eesti Kutseliste Mesinike Ühing MTÜ liige

2017-… Simmu Mesi OÜ juhatuse esimees

2019-2023 Horizon2020 projekt "PoshBee," EKMÜ koordinaator

2020-2022 Projekt "Tarukaalude andmeanalüüs Eesti Mesindusprogrammi 2019–2020 raames" (1.06.2020–30.04.2022), projektijuht

2019-2022 Mesindusprogrammi 2020–2022 alameede A8 (Iseseisvate tarukaalude haldamine, analüüs), alameetmejuht

2022- … Tarukaalude koordineerimine, andmeanalüüs

LIST OF PUBLICATIONS

Publications indexed in the ISI Web of Science database:

Naudi, S., Steiselis, J., Jurison, M., Raimets, R., Tummeleht, L., Praakle, K., Raie, A., Karise, R. (2021). Variation in the distribution of *Nosema* species in honey bees (*Apis mellifera* Linnaeus) between the neighboring countries Estonia and Latvia. Veterinary Sciences, 8(4). DOI: 10.3390/vetsci8040058

Naudi, S., Rossi, F., Ricchiuti, L., Manocchio, P., Del Matto, I., Jürison, M., Viinalass, H., Karise, R., Raimets, R. (2025). Could hive debris samples and qPCR ease the investigation of factors influencing *Paenibacillus larvae* spore loads? DOI: 10.1080/00218839.2024.2435222.

Jürison, M., Pent, K., Raimets, R., Naudi, S., Mänd, M., Karise, R. (2025). Azoxystrobin hides the respiratory failure of low-dose sulfoxaflor in bumble bees. Ecotoxicology and Environmental Safety, 289, 117487. DOI: 10.1016/j.ecoenv.2024.117487.

Raimets, R., Naudi, S., Bartkevics, V., Pugajeva, I., Mänd, M., Karise, R. (2019). Translocation of tebuconazole between bee matrices and its potential threat on honey bee (*Apis mellifera* Linnaeus) Queens. Insects, 13(1):45. DOI: 10.3390/insects13010045

Pent, K., Naudi, S., Raimets, R., Jürison, M., Liiskmann, E., Karise, R. (2023). Overlapping exposure effects of pathogen and dimethoate on honey bee (*Apis mellifera* Linnaeus) metabolic rate and longevity. Frontiers in Physiology, 14. DOI: 10.3389/fphys.2023.1198070

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Naudi, S., Raimets, R., Jürison, M., Liiskmnn, E., Mänd, M., Salkova, D., Karise, R. (2022). Effect of Nosema apis and *N. ceranae* on honey bee *Apis mellifera* queen development. Acta Morphologica et Anthropologica, 29 (3-4). DOI: 10.7546/AMA.29.3-4.2022.33

Salkova, D., Panayotova-Pencheva, M., Naudi, S. (2024). Honey bee diseases and their control in Bulgaria and Estonia: a review. Tradition & Modernity in Veterinary Medicine, 2024. DOI: 10.5281/zenodo.12744122

3.2. Papers published in books by Estonian or foreign publishers not listed in the ISI Web of Proceedings

Raimets, R., Karise, R., Mänd, M., Naudi, S., Bontšutšnaja, A., Cresswell, J. (2017). Pestitsiidide segud on karukimalastele (*Bombus terrestris* L.) üksikute ainete mõjudest ohtlikumad. Teaduselt mahepõllumajandusele. (130–134). SA Eesti Maaülikooli Mahekeskus. **Naudi**, S., Bontšutšnaja, A., Raimets, R., Karise, R. (2017). Mesinduse roll ning olulisus mahemesinduse perspektiivis. Teaduselt mahepõllumajandusele. (105–112). Tartu 2017:SA Eesti Maaülikooli Mahekeskus.

Bontšutšnaja, A., Raimets, R., Naudi, S., Karise, R. (2017). Kimalasperede areng sõltub suurel määral korjevõimaluse olemasolust ja vähem ümbritsevast taimekooslusest. Metspalu, Luule; Luik, Anne; Peetsmann, Elen (Toim.). Teaduselt mahepõllumajandusele. (19–23). Tartu: SAEesti Maaülikooli Mahekeskus.

Naudi, S., Kullman, B., Tummeleht, L., Jürison, M., Raimets, R., Šteiselis, J., Karise, R. (2019). Mikrosporiidsed parasiidid *Nosema apis* & *Nosema* ceranae meemesilastes – muutus ajas ning liigiline jaotus. Metspalu, Luule; Luik, Anne (Toim.). Teaduselt mahepõllumajandusele. (82–88). SA Eesti Maaülikooli Mahekeskus.

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3.5. Articles/presentations published in local conference proceedings

Pent. K., Raimets, R., Naudi, S., Jürison, M., Karise, R. (2022). Õietolmubotaaniline päritolu ühe taimekasvatusperioodi jooksul Eesti mesilates ja põllukultuuride osakaal nendes. Maarika Alaru (Toim.). AGRONOOMIA 2022. (162–168). Vali press.

6.3. Popular science articles

Naudi, S. (2018). Nosemoos, haigusega toimetulek ning levik Eestis. Mesinik, 11–13,01.04.2018.

VIIS VIIMAST KAITSMIST

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Professor Andres Valdmann
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VIVIAN KUUSK
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LAMMASTE HEAOLU VÕRDLEV HINDAMINE ERINEVATES PIDAMISSÜSTEEMIDES
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Professor David Arney, lektor Peep Piirsalu
9. jaanuar 2025

DANIELA GHIA
INVASIIVSETE VÄHILIIKIDE KÄITUMISOMADUSTE JA ÖKOLOOGILISE MÕJU HINDAMINE MAGEVEEKOGUDES
ASSESSING BEHAVIOURAL TRAITS AND ECOLOGICAL IMPACTS OF INVASIVE CRAYFISH SPECIES IN FRESHWATER ECOSYSTEMS
Teadur Fabio Ercoli, professor Roberto Sacchi
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