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Study of factors influencing viral infections in honey bees

Ph.D. Thesis

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Annotation:

This thesis includes three published articles, two of which are first-authored. The first article demonstrates the antiviral effect of *Cortinarius caperatus* alcohol extract against Deformed wing virus in honey bees, its influence on the transcriptional activity of selected immune genes, and its impact on honey bee lifespan. The second article explores differences in gut bacterial networks between Swedish varroa-surviving and local varroa-susceptible honey bees, as well as correlations between these bacteria and pathogenic viruses. The third article describes the CO_2 conductance and microstructure of wax cappings, along with CO_2 gradients resulting from pupal respiration. Altogether, the work presented in this thesis extends the repertoire of potential treatments against honey bee viruses, provides new insights into honey bee gut bacterial communities, and lays a foundation for future research on mechanisms contributing to varroa resistance.

Declarations

I hereby declare that this Ph.D. thesis is my own original work and that I have used only the sources and literature listed in the references. Language optimization was performed with the assistance of the language model ChatGPT (GPT-5).

Place & Date: České Budějovice, 3.11. 2025 **Karolína Svobodová**

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INTRODUCTION

1. Importance of honey bees

Since the dawn of human history, honey bees have been closely connected with humankind, as "*honey hunting by man is as old as man himself*" (Crane, 1999). As early as *Australopithecus*, *Homo habilis* and *Homo erectus*, hominids exploited wild honey bee colonies as a source of dietary proteins and carbohydrates (Crane, 1999). Members of early human hunter-gatherer communities harvested honey from trees and rock outcrops during their hunting trips, as illustrated in Mesolithic cave paintings, the oldest of which was found in Valencia, Spain (Crane, 1999). The first evidence of apiculture can be traced to ancient Crete, Egypt and the Levant, where people managed honey bee colonies in wooden boxes or clay cylinders that enabled a controlled production of bee products. In the Middle Ages, apiculture prospered because honey bee products were significant trade articles and honey bees were managed in hives of various shapes and materials (Crane, 1999). For instance, beekeepers from Central and Eastern Europe often managed their honey bees in artificially modified tree cavities referred to as forest beekeeping (Ilyasov et al., 2024). In 1622, European honey bees were introduced to North America by settlers, followed by their introduction to Australia in 1822 and to New Guinea and the Pacific Islands in 1857.

Honey bee products have served many purposes throughout history, and many of these uses are similar today. Honey has been consumed as food, used as a sweetener, and fermented to produce alcoholic drinks. Besides its dietary value, honey has been used in medicine and cosmetics. Additionally, honey had ritual significance in some ancient cultures. For instance, honey was used in rituals associated with the afterlife and fertility in ancient Egypt (Bowie 2020). Beeswax was an essential material for the production of candles widely used for lighting before the advent of electricity. Moreover, it was also commonly used for sealing documents, polishing wood and leather, and forming molds for metal casting (Crane, 1999). Even though cheaper alternatives to honey bee products nowadays exist, they are still popular and highly desirable. For instance, the survey of honey consumption in the Polish population from 2022 showed that about 40% of respondents consume honey either daily or at least several times per week, most often in hot drinks or in sandwiches (Kowalczuk et al., 2023).

Besides generating honey bee products, honey bees also hold importance as pollinators. In agriculture, insect pollination plays a significant role in plant production, and its global economic value was estimated at 153 billion per year (Gallai et al., 2009). Honey bees are the

most frequent pollinator species, and their contribution has been shown to increase the yields of some crops, fruits, and vegetables. For example, Chautá-Mellizo et al. (2012) showed that bee-pollinated gooseberry plants produced larger fruits with higher seed germination rates than those that were manually or self-pollinated. Pollination by honey bees also significantly increases the fruit set of cotton as well as the fruit and seed weight of sesame (Stein et al., 2017). Additionally, Bartomeus et al. (2014) showed that open pollination dominated by honey bees promoted increased yield of oilseed rape, buckwheat, and strawberry. However, effective agricultural pollination requires a whole range of pollinators, because honey bees alone are not as effective as a diverse assemblage of pollinators (Garibaldi et al., 2013). That can be exemplified by the study of Mallinger et al. (2015), who investigated the pollination of apple trees. They showed that honey bees tend to prefer trees with a greater abundance of blossoms and often concentrate their activity in the upper canopy. On the other hand, wild bees displayed a more randomized visitation pattern, resulting in a correlation of the production of apples with pollinator species richness. Alongside other pollinators, honey bees also participate in the pollination of wild floral species (Hung et al., 2018).

Despite their importance, honey bees face serious threats resulting in substantial colony losses, reaching up to nearly 50% of honey bee colonies kept in the USA (Bruckner et al., 2023) and about 20% in Europe (Gray et al., 2020; Gray et al., 2022). These losses present a significant economic impact, calculated at up to tens of millions of euros in some European countries in 2017 (Popovska Stojanov et al., 2021). Although the mortality of honey bee colonies occurs in each season, conditions of winters in temperate regions, such as the lack of food sources and low temperatures, make this season significant from the point of colony losses. Furthermore, at the beginning of the 21st century, a new type of colony mortality called colony collapse disorder (CCD) appeared (van Engelsdorp et al., 2007). The CCD is characterized by rapid disappearance of adults, and the affected colony often remains with supplies and brood with a minimal number of workers, if any (van Engelsdorp et al., 2009). These losses are caused by a combination of multiple factors such as the use of pesticides, poor nutritional status, climate change, and the spread of parasites and diseases (Goulson et al., 2015; Hristov et al., 2020). However, a parasitic mite, *Varroa destructor*, has a prominent role in honey bee colony losses (Dahle, 2010; van Dooremalen et al., 2012), and the level of varroa mite infestation has been shown as a strong predictive marker for colony mortality (Dainat et al., 2012). The following chapters will research the biotic threats to honey bee colonies and mechanisms of protection against them, performed either naturally by the bees or artificially by beekeeping interventions.

2. Parasites and pathogens of honey bees

2.1. Parasitic mite Varroa destructor

The parasitic mite *Varroa destructor* is currently one of the most significant biotic threats to honey bee populations worldwide (Traynor et al., 2020). Originally, this mite parasitized the eastern honey bee, *Apis cerana*, which evolved effective mechanisms to combat varroa mite infestations (Grindrod & Martin, 2023). However, during the second half of the 19th century, the western honey bee, *Apis mellifera*, was introduced to eastern Asia, where it encountered the eastern honey bee and its parasites (Bruce Krejčí et al., 2023). Consequently, the varroa mite successfully shifted hosts to western honey bee and began to cause significant harm, as only small proportion of western honey bee colonies possess the ability to defense against the parasite. This host switch enabled the mite to spread across most of the world during the second half of the 20th century. By 2022, it had reached the last remaining continent, Australia. Today, the only varroa free regions where honey bees are kept are isolated islands.

Genetic mapping of the mite revealed that two independent host-switching events occurred in East Asia, giving rise to two lineages of varroa mites infesting the western honey bee (de Guzman et al., 1997; Oldroyd, 1999), the Korean haplotype and the Japanese haplotype. While the Korean haplotype is almost globally prevalent, the Japanese haplotype has been detected only in Japan, Thailand, and parts of the Americas (Solignac et al. 2005; Traynor et al., 2020). The mites of the two haplotypes hybridize in regions where they co-occur (Solignac et al. 2005; Hua et al., 2023). Interestingly, differences between the two haplotypes have been identified not only at the genetic level (Morfin et al., 2023) but also in their cuticular hydrocarbon profiles (Nation et al., 1992). However, due to the bottleneck effect during the host shift, as well as founder effects and the pseudo-arrhenotokous haplodiploid mode of reproduction, varroa mites display low within-haplotype genetic diversity (Rosenkranz et al., 2010). Despite the low diversity, detectable genetic variations exist between apiaries and colonies (Dynes et al., 2017), which likely enables varroa mites to resist inbreeding depression, as the infestation of a single brood cell by multiple foundress mites increases the recombination rate and the level of heterozygosity in varroa populations (Beaurepaire et al., 2017).

2.1.1. Lifecycle of Varroa destructor

The lifecycle of the varroa mite consists of reproductive and dispersal phases, which are closely associated with the development of honey bees (Rosenkranz et al., 2010). The

reproductive phase begins when a foundress mite locates a larva of the last instar and invades the brood cell of a drone or worker approximately 40 and 20 hours, respectively, before capping (Boot et al., 1992), in which it hides in the brood food until the sealing of the cell (Ifantidis, 1998). Then, the foundress female mite bites the honey bee pupa usually on the fifth abdominal sclerite to create a feeding site for her and her future progeny (Donzé & Guerin, 1994). The anticoagulant compounds of its saliva inhibit hemocyte aggregation, therefore the wound remains open during the metamorphosis of the honey bee (Han et al., 2024; Richards et al., 2011). In addition to providing nutrition, the feeding on honey bee pupa also provides ecdysone to the foundress mite, which is required for the initiation of oviposition, but the mite cannot produce it on its own (Conlon et al., 2019). Approximately 60 to 70 hours after the brood cell is sealed, the foundress mite lays a single haploid male egg and later diploid female eggs. In worker brood cells the foundress can lay up to four female eggs and up to seven in drone brood cells, with intervals of approximately 30 hours between each oviposition (Nazzi & Le Conte, 2016). The newly hatched progeny advance through the protonymph and deutonymph stages. Upon reaching adulthood, the male fertilizes the newly hatched females (Donzé & Guerin, 1994; Ziegelmann et al., 2013). The fertilized female varroa mites then leave the cell with the newly emerged honey bee, while the male varroa mite dies inside the brood cell. The reproductive stage is over, and the dispersal stage begins at this point. In the dispersal phase, female varroa mites locate and attach to a nurse honey bee, which subsequently transports them to brood cells to initiate a new reproductive phase. Mites may favor attaching to forager honey bees in colonies with high varroa populations (DeGrandi-Hoffman et al., 2016), subsequently spreading them to other honey bee colonies. This phase usually lasts about 7 days (Boot et al., 1993; Harbo & Harris, 1999; Sammataro et al., 2000) and is probably necessary for the maturation of spermatozoa stored in the varroa female after mating. Häußermann et al. (2016) demonstrated that spermatozoa capacitation in the varroa mite spermatheca requires at least 5 days, and mites with a restricted dispersal phase had significantly reduced fertility. However, varroa mites that experience a prolonged dispersal phase due to an interruption of brood production are also less fertile (Gabel et al., 2023). This implies that a dispersal phase of the appropriate duration is necessary for optimal varroa mite fertility.

2.1.2. Effects of varroa infestation on honey bees

Varroa mite infestation is one of the most significant causes of honey bee colony losses worldwide. The deadly potential of this mite stems mainly from its association with severe viruses; however, feeding on honey bee tissues by the varroa mite also impairs host health. Han et al. (2024) showed that varroa mites in the reproductive stage feed on pupal fat bodies and a considerable amount of hemolymph. The loss of hemolymph and fat body aligns with the observation by Bowen-Walker & Gunn (2001) that honey bees emerged from varroa-infested cells have reduced weight and content of water and proteins. In adult honey bees, varroa feeding is associated with alternations in cuticular hydrocarbon profiles (Bowen-Walker & Gunn, 2001; Annoscia et al., 2012) and decreased numbers of hemocytes, which is associated with reduced responses to cellular immunity (Koleoglu et al., 2018; Morfin et al., 2020). In addition, the wounds caused by varroa were shown to contain bacterial colonies (Kanbar & Engels, 2003) that could possibly cause bacterial infiltration and consequent infection. Uroš et al. (2014) detected the honey bee gut parasite *Nosema ceranae* in the hemolymph of varroa-infested honey bees. However, varroa mite is an effective vector of honey bee viruses including Deformed wing virus, AKI complex viruses, and likely Sacbrood virus (Traynor et al., 2020; Shen et al., 2005).

2.1.3. Impact of the *Varroa destructor* on the honey bee virome

The global spread of varroa mites markedly changed the original profiles of honey bee viruses. A general trend observed by multiple studies indicates that the arrival of the varroa mite into new areas reduces the diversity of total viruses and increases loads of certain few viruses, mainly DWV. For instance, after the arrival of varroa mites in the Hawaiian Islands, increased DWV prevalence went from 13% up to 75%–100%, and DWV loads were about a millionfold higher (Martin et al., 2012). Furthermore, the local unique DWV strains were suppressed by a single dominant DWV strain (Martin et al., 2012). The decline in viral diversity was also observed by Doublet et al. (2024), who analyzed honey bee samples from multiple sites across the world after and before the varroa mite spread. Notably, they identified BQCV and SBV as the most prevalent viruses before the establishment of the varroa mite; after that, the prevalence of BQCV, SBV, and CBPV increased. However, the increase in DWV was so significant that it surpassed the other viruses and became dominant (Doublet et al., 2024). Interesting differences in viral diversity were also shown by Kadlečková et al. (2022)

comparing the presence of viruses in honey bees from varroa-free Australia and the Czech Republic, where the varroa mites were present. While honey bees from Australia contained several tens of viruses without a trace of DWV, the honey bees from the Czech Republic harbored only nine viruses, with DWV being the most prevalent. Together, these studies indicate that the associations of varroa mite with certain viruses, mainly DWV, reshape viral communities in honey bee populations.

2.2. Honey bee viruses

Currently, over 70 viruses have been detected in honey bees, but only 12 of them were identified as causative agents of honey bee health issues (Beaurepaire et al., 2020). Most of these pathogenic viruses are single-stranded RNA viruses from the Picornavirales order, specifically the Iflaviridae and Dicistroviridae families, apart from the DNA virus Apis mellifera filamentous virus and the unclassified Chronic bee paralysis virus. These viruses have similar structures and genomes (Chen et al., 2012). They are mostly non-enveloped, with isometric capsids composed of three or four VP subunits. The genome of these viruses is composed of a monopartite positive-sense 8 to 10 kb long single-stranded RNA. The genome of Iflaviridae viruses contains a single ORF coding for structural proteins, RNA helicase, chymotrypsin-like 3C protease, and RNA-dependent RNA polymerase. In contrast, the genome of Dicistroviridae honey bee viruses contains two non-overlapping ORFs separated by a UTR. The 3' ORF encodes capsid proteins, while the 5' ORF encodes a nonstructural precursor that is auto-proteolytically cleaved into viral enzymes.

The propagation mode is similar for both Iflaviridae and Dicistroviridae viruses (Chen et al., 2012; Louten, 2016). For entry into the cell, the virus binds to a surface cell receptor that triggers the formation of endosomes carrying the virus inside the cell. The specific receptors used by honey bee viruses are not yet known. Low pH in the endosome triggers conformational changes in the viral capsid proteins that lead to the release of the viral genome into the cytoplasm (Škubník et al., 2021). The viral RNA is then directly translated into a polyprotein and also transcribed into a complementary negative strand used as a template for the transcription of the new viral genome. The RNA genome is then covered by viral proteins, and the new viral particle is assembled. Because the process of viral replication depletes the resources of the cell, the translation of cellular material is inhibited, and infected cells cannot properly function. Finally, the infected cell lyses and the new viral particles are released.

2.2.1. Most significant honey bee viruses

2.2.1.1. Deformed wing virus

DWV is currently the most prevalent virus in western honey bee populations and is considered to be at epidemic levels (de Miranda & Genersch, 2010). DWV is a viral quasispecies in the Iflaviridae family consisting of three closely related strains. While the two of them, the DWV-A and DWV-B are widely spread across the world, DWV-C was detected only rarely (de Miranda et al., 2022). In the initial phase of varroa mite global invasion was characterized by the spread of DWV-A variant mechanically vectored by the mite. However, in recent years, this variant has been replaced by DWV-B, likely because of its lower virulence and the ability to replicate in varroa mites, which may favor its dissemination (Norton et al., 2020; Barth et al., 2024; Sircoulomb et al., 2025; Gisder et al., 2021). The virus causes either covert or overt infections, depending on the mode of its transmission (de Miranda & Genersch, 2010). The covert infections are characterized by low viral loads with no visible symptoms. This type of DWV infection usually occurs when the virus is transmitted vertically by queens and drones or horizontally through ingestion of contaminated food or cannibalization of infected individuals (de Miranda & Genersch, 2010; Posada-Florez et al., 2021). However, even in asymptomatic honey bees, the virus replicates in their gut epithelium (Fievet et al., 2006). Correspondingly, covert DWV infections trigger responses of cellular immunity in midgut tissues including infiltration by plasmatocytes, granulocytes, and melanin deposition (Power et al., 2021). On the other hand, overt infection emerges after the DWV is vectored by varroa mites to honey bee pupae and typically progresses in high viral levels (Tentcheva et al., 2006). The infection manifests characteristic symptoms such as wing deformities, bloated abdomens, body discoloration, and increased mortality (de Miranda & Genersch, 2010). Yue & Genersch (2005) demonstrated that varroa mites in honey bee colonies carry varying levels of DWV, therefore the parasitism of individual mites leads to varying severity of infection in individual honey bees. Consensually, Ryabov et al. (2022) found that only about 40% of varroa mites induced overt symptoms in honey bees for both DWV-A and DWV-B, and that their infectivity increases after a prolonged phoretic stage. Adult honey bees injected with DWV, mimicking infection from phoretic mites, do not develop visible symptoms but exhibit reduced lifespan, impaired learning and memory disabilities, earlier foraging and reduced foraging performance (Benaets et al., 2017; Chen et al., 2021). The loss of honey bee workers, along with a high proportion of non-productive, diseased individuals and the reduced lifespan of winter honey bees, leads to weakening and losses of colonies (de Miranda & Genersch, 2010;

Dainat et al., 2012; Highfield et al., 2009; Francis et al., 2013; Barroso-Arévalo et al., 2019; Dainat & Neumann, 2013; Natsopoulou et al., 2017).

2.2.1.2. Black queen cell virus

Black queen cell virus (BQCV) is a globally distributed virus (Ellis & Munn, 2015) belonging to the *Triatovirus* genus within the *Dicistroviridae* family. It has an icosahedral capsid and is larger than most other honey bee viruses (Spurny et al., 2017). BQCV primarily affects honey bee queen larvae and pupae, kills them and causes black color of their cadavers (Bailey & Woods, 1977). However, the virus is also frequently detected in adult workers (Muz & Muz, 2017), drones (Phokasem et al., 2021) and queens, particularly in their guts and ovaries (Chen et al., 2006). The virus spreads through both vertical and horizontal transmission, it can be transmitted via eggs from infected queens to their offspring, as well as through food and possibly via feces (Chen et al., 2006; Singh et al., 2010). Al Naggar & Paxton (2020) found that while oral ingestion of BQCV does not significantly shorten the lifespan of worker bees, direct injection of the virus into the hemolymph mimicking its transmission via vector leads to a significant reduction in longevity of honey bees. However, there is currently no evidence of BQCV transmission through varroa mites (Yañez et al., 2020). Interestingly, BQCV is often associated with microsporidium *Nosema apis* (Bailey et al., 1983). Although this co-occurrence raises the possibility of a synergistic interaction, further research is needed to clarify the exact relationship between these two pathogens and their combined impact on honey bee populations.

2.2.1.3. Sacbrood virus

Sackbrood virus (SBV) is a globally distributed honey bee virus belonging to the Iflaviridae family (Wei et al., 2022). It has an unusual icosahedral capsid with a spherical shape (Procházková et al., 2018) and a ssRNA genome (Ghosh et al., 1999). SBV infects all honey bee castes and developmental stages, but larvae are the most severely affected. Infected larvae fail to molt that leads to the accumulation of fluid between the body and the unshed cuticle resulting in typical sac-like appearance. As the infection progresses, the larvae change color from white to yellow and turn brownish after death (Wei et al., 2022). In adult honey bees, SBV does not exhibit visible symptoms, but the virus can accumulate in the head and affect foraging behavior and food preferences (Bailey & Fernando, 1972). The virus spreads through

both vertical and horizontal transmission. Infected queens can transmit SBV to their offspring via eggs, while horizontal transmission occurs through contaminated food (Shen et al., 2005). Additionally, the varroa mite serves as a mechanical vector for SBV (Shen et al., 2005; Tentcheva et al., 2004). SBV outbreaks are seasonal, and infection peaks usually occur in spring and summer (Tentcheva et al., 2004).

2.2.1.4. AKI complex viruses

The AKI complex consists of three closely related viruses from the Dicistroviridae family, Acute bee paralysis virus (ABPV), Kashmir bee virus (KBV), and Israeli acute bee paralysis virus (IAPV) (de Miranda et al., 2010). These viruses share a monopartite, bicistronic genome organization and are globally distributed. ABPV has been reported on all continents where honey bees are found, while KBV is prevalent in North America, New Zealand, and Australia, and IAPV is detected mainly in America and the Middle East (de Miranda, 2010; Ellis & Munn, 2015; Beaurepaire et al., 2020). Transmission occurs through multiple routes, including oral ingestion (Maori et al., 2009; Chen et al., 2004), vertical transmission from queens and drones to offspring (Ravoet et al., 2015; Chen et al., 2004), and vector mediated transmission via varroa mites (Chen et al., 2004; Shen et al., 2005). These viruses can persist as covert infections without visible symptoms (Anderson, 1991), but more often cause a rapid mortality of infected honey bees that makes clinical symptoms difficult to detect (de Miranda et al., 2010). Notably, when directly injected, these viruses exhibit high virulence and can kill honey bees within a few days (Maori et al., 2007; Bailey et al., 1963; Dall, 1987). The AKI complex is frequently associated with high varroa mite infestations and is commonly detected in weakened or collapsing colonies (Hou et al., 2014; Bakonyi et al., 2002; Chen et al., 2014). Furthermore, Chen et al. (2014) demonstrated that IAPV infects all honey bee castes and sexes in all developmental stages and it replicates in multiple honey bee tissues, including the brain, nervous system, gut, fat body, salivary glands, hypopharyngeal glands, and muscles.

2.2.1.5. Chronic bee paralysis virus

Chronic bee paralysis virus (CBPV) is an unusual honey bee virus related to the *Nodaviridae* and *Tombusviridae* families, though it remains further unclassified. The virus has an anisometric, ellipsoidal structure (Bailey et al., 1968) and a multipartite genome consisting of two major RNAs (Olivier et al., 2008), which are translated into four polypeptides (Ribière et

al., 2000). Interestingly, CBPV is accompanied by a satellite virus known as Chronic bee paralysis virus satellite (Bailey, 1967; Fernandez de la Mora et al., 2020). CBPV infection manifests in two distinct phenotypes. Honey bees having the first CBPV phenotype exhibit symptoms of paralysis, including bloated abdomens, body and wing trembling, and inability to fly, while the second phenotype manifests by loss of hairs causing a black, shiny appearance of infected honey bees (Bailey, 1967). These individuals are typically able to fly but display increased aggression toward their nestmates (Rinderer & Rothenbuhler, 1976). In both cases, infected honey bees die shortly after symptom onset. The virus can be detected in all body parts of diseased honey bees and primarily targets the brain, ganglia, and nerves (Ribière et al., 2010). It is globally distributed and, although it exhibits a seasonal pattern with overt infections typically occurring in summer, outbreaks have also been observed in winter (Ribière et al., 2010).

2.2.1.6. Lake Sinai virus

Lake Sinai virus (LSV) is a monophyletic complex comprising seven recently identified strains. It belongs to the *Sinhaliviridae* family within the order *Nodamivirales* and possesses a ssRNA genome. LSV has been detected in America, Europe, and China (Cepero et al., 2014; Daughenbaugh et al. 2015; Čukanová et al., 2022; Hou et al., 2023). The pathology of LSV remains unclear, and different strains may have varying impacts on honey bee health. However, LSV has been associated with weak and collapsing colonies (Hesketh-Best et al., 2024; Faurot-Daniels et al., 2020; Cepero et al., 2014), suggesting a potential role in colony decline. The exact effects on honey bee physiology are still unknown, but Daughenbaugh et al. (2015) detected the highest levels of LSV in the honey bee gut, followed by the thorax, with lower levels present in the head. Additionally, LSV was detected in varroa mites (Daughenbaugh et al., 2015; Shojaei et al., 2021), indicating the potential for vector-mediated transmission. Beyond honey bees, LSV has also been identified in wild hymenopteran species (Ravoet et al., 2014; Šimenc et al., 2020), suggesting that the virus may spread through contaminated pollen.

2.3. Bacterial pathogens

Paenibacillus larvae is a gram-positive, spore-forming bacterium that causes American Foulbrood (AFB). While adult honey bees remain unaffected, the disease specifically targets

larvae, so it disrupts worker turnover and leads to colony collapse. The bacterium spreads within the hive attached to adult bees, which then transmit it to the brood. Larvae around 24 hours old are the most susceptible, and fewer than 10 spores are sufficient to initiate infection (Woodrow, 1942; Brødsgaard et al., 1998). Once ingested, the spores germinate in the larval gut, utilize available nutrients, and initially proliferate harmlessly. Since young larvae have a thin peritrophic membrane lacking chitin, *P. larvae* penetrates the gut epithelium (Davidson, 1973). In the later stages of infection, the bacterium produces proteolytic enzymes that facilitate its entry into the hemocoel, and consequent systemic infection is followed by a rapid larval death. The cadaver is then degraded into a viscous, brown substance known as "ropy mass," which adheres to the brood cell walls and releases billions of newly formed spores upon drying (Genersch, 2010). *P. larvae* exhibits different genotypes and biochemical variations that affect the progression of infection. Based on enterobacterial repetitive intergenic consensus (ERIC) classification, five strains of *P. larvae* have been identified (Beims et al., 2020). These strains differ in their phenotypic effects. ERIC I kills infected larvae within 12–14 days, while ERIC II causes larval death within 6–7 days. Additionally, larvae infected with ERIC I are less frequently removed from the colony and contribute to up to four times higher spore production compared to those infected with ERIC II (Rauch et al., 2009). ERIC III, IV, and V are rare and thus epidemiologically insignificant (Morrissey et al., 2015). ERIC I is the most widespread variant globally (Morrissey et al., 2015), whereas ERIC II is the dominant strain in the Czech Republic (Biová et al., 2021).

Another significant bacterial pathogen is *Melissococcus plutonius*, a Gram-positive bacterium responsible for the devastating larval disease European foulbrood (EFB). While adult honey bees remain unaffected, they transmit the bacterium to the brood. The colonization process of *M. plutonius* was well described by Takamatsu et al. (2016), who artificially infected honey bee larvae and examined the resulting histopathological changes. Infection begins when larvae ingest contaminated food, allowing the bacterium to proliferate in the gut contents. In the early stages, *M. plutonius* remains confined to the food mass but later penetrates the gut epithelium. After 4–5 days, it melts the peritrophic membrane, invades gut epithelial cells, and the infected larva dies. At this stage, secondary invaders such as *Paenibacillus alvei* and *Enterococcus faecalis* often colonize the infected larvae (Bailey et al., 1973). Although *M. plutonius* can be detected in deeper tissues, it likely does not proliferate there (Takamatsu et al., 2016). Affected larvae turn from yellow to brown, develop a melted appearance, and release a characteristic foul odor. *M. plutonius* has been categorized into three clonal complexes with varying virulence: CC12 is highly virulent, CC3 is moderately virulent, and CC13 is avirulent (Pérez-

Ordóñez et al., 2021). However, the virulence of *M. plutonius* appears to be strongly influenced by the genetic background of its host and exposure to pesticides (Lewkowski & Erler, 2019; Thebeau et al., 2023). This bacterium is globally distributed and can be detected in asymptomatic colonies near infected ones (Alburaki et al., 2024; Grossar et al., 2023; Pinnock & Featherstone, 2015).

2.4. Microsporidian, fungal and protozoan pathogens

Honey bee colonies are vulnerable to various pathogens, including microsporidia, fungi, and protozoan parasites. These pathogens can negatively impact colony health, sometimes contributing to colony collapse. Two microsporidian species, *Nosema apis* and *Nosema ceranae*, primarily infect the gut tissues of honey bees and are transmitted through the fecal-oral route (Higes et al., 2020). The life cycle of *Nosema* begins when spores use a polar tube to inject sporoplasm into a host cell. The sporoplasm develops into a meront, which divides into paired meronts. These then differentiate into sporonts, mature into sporoblasts, and form new spores (Galajda et al., 2021). The spores are oval-shaped, with *N. apis* measuring approximately 6 × 3 µm and *N. ceranae* around 5 × 3 µm (Ptaszyńska et al., 2015). The effects of these two species on colonies differ. *Nosema apis* infections are seasonal, occur mainly in winter and cause diarrhea and fecal staining on hive equipment. In contrast, *N. ceranae* is present year-round without visible symptoms (Galajda et al., 2021). *N. apis* is likely the original parasite of the western honey bee, while *N. ceranae*, originally a parasite of the eastern honey bee, has likely switched hosts and is now replacing *N. apis* globally. Long-term monitoring in Germany confirmed that *N. ceranae*, not *N. apis*, plays a role in colony collapses (Schüler et al., 2023). Although *N. ceranae* has been frequently detected in weak and collapsing colonies (Higes et al., 2009; Botías et al., 2013), Schüler et al. (2023) found that its statistical impact on colony collapse is relatively minor, despite its negative effects on honey bee health.

In addition to microsporidia, fungal infections can also threaten colony health. *Ascosphaera apis* is a fungal pathogen that infects honey bee larvae and pupae, and manifest by typical mummy-like appearance in affected individuals (Deneke et al., 2023). Although adult bees are not directly affected, infected colonies experience worker losses leading to reduced colony strength and lower honey yields (Zaghloul et al., 2005). The infection is promoted by

decreased temperatures and increased humidity (Flores et al., 2006). In temperate regions, outbreaks typically occur in spring and early summer (Deneke et al., 2023).

Protozoan parasites, such as *Lotmaria passim*, *Crithidia mellificae*, and *Malpighamoeba mellificae* also pose potential risks to honey bees. While the trypanosomatids *L. passim* and *C. mellificae* infect the honey bee gut, amoeba *M. mellificae* targets epithelia of Malpighian tubules. Laboratory testing has shown that the damage caused by trypanosomatids shorten honey bee lifespan (Gómez-Moracho et al., 2020). Due to these harmful effects, they are suspected contributors to colony losses (Ravoet et al., 2013). However, their role in colony health remains unclear, as they are widespread but not always associated with colony decline.

3. Immunity of honey bees

Like other arthropods, honey bees lack the adaptive immunity found in vertebrates and thus rely on innate immunity, which provides rapid but less specific responses. Their immune system operates on multiple levels, including physical barriers as well as cellular and humoral mechanisms that cooperate to combat intruders. Moreover, as social insects, honey bees have evolved collective defense mechanisms, referred to as social immunity, that help prevent and mitigate the spread of parasites and diseases within the colony. Interestingly, genomic studies have revealed that although honey bees share conserved immune mechanisms with other insects, their repertoire of immune genes is only about one-third the size of that found in *Drosophila* and mosquitoes (Evans et al., 2006, Elsik et al., 2014). This reduction is likely a consequence of eusociality, where the evolution of social immunity may have allowed for the reduction of certain components of costly individual immunity (Evans et al., 2006). The following paragraphs will discuss the immune mechanisms of honey bees, including both individual and social immunity.

3.1. Immunity at individual level

3.1.1. Physical barriers

The first layer of defense protecting organisms from intruders consists of physical barriers. In insects, a major physical barrier is the cuticle, an apical extracellular matrix produced by the epidermis, tracheal epithelium, and the epithelial linings of the foregut and hindgut (Li Zheng et al., 2020). The cuticle is composed of multiple layers of chitin and proteins, making it thick, durable, and therefore difficult to penetrate for pathogens (Neville, 1975). Additionally, the cuticle provides biochemical protection, as damaged areas release antimicrobial compounds (Brey et al., 1993). Microorganisms can enter the integument by infiltration of wounds on the body surface, such as punctures caused by varroa mite feeding (Kanbar & Engels, 2003). Some viruses are also directly transferred to the honey bee organism in the saliva of varroa mites (Shen et al., 2005; Zhang & Han, 2019). The midgut produces a specialized type of cuticle, the peritrophic membrane (Teixeira et al., 2015). Although it is also composed of chitin and proteins, its porous and permeable structure allows the absorption of nutrients (Lehane, 1997). The gut environment is also protected by the activity of gut-resident microbes, whose actions cause acid pH and antagonize invading bacteria (Steele et al., 2021).

After microorganisms manage to enter the internal environment, they are recognized by the immune system using several Pattern Recognition Receptors (PRRs). These receptors bind to Pathogen-Associated Molecular Patterns (PAMPs), molecular signatures unique to pathogens and absent in the host (Wang et al., 2019). Several types of PRRs were found in honey bees, including peptidoglycan recognition proteins (PGRPs), β-glucan recognition receptors, galectins, C-type lectins, fibrinogen-related proteins, DSCAM, and scavenger receptors (Evans et al., 2006). Upon recognition of injury and foreign molecular patterns, the organism firstly reacts to plug the wound via clot formation, then triggers cellular responses, and finally produces humoral factors.

3.1.2. Coagulation

Injured organism must respond rapidly to minimize microbial invasion and loss of hemolymph. This is particularly important in larvae, where hemolymph is under pressure to maintain a hydroskeleton (Wigglesworth, 2012). The initial response of an insect to injury is coagulation mediated by hemocytes and hemolymph coagulogen. First, a soft primary clot forms, which is then hardened and cross-linked (Aprelev et al., 2019; Strand, 2008). This process is performed by a coagulation cascade that, in *Drosophila*, involves the enzyme transglutaminase, which catalyzes covalent linkages between glutamine and lysine residues. Key components of coagulation include hemolectin, lipophorin, phenoloxidase, and hexamerins (Theopold et al., 2014). Although the exact mechanism in honey bees was not extensively studied, the identification of the hemolectin gene in their genome implies that a similar process likely occurs (The Honeybee Genome Sequencing Consortium, 2006; Lesch et al., 2007). Clotting is coupled with melanization and the phenoloxidase cascade, which contributes to clot hardening and the elimination of microorganisms infecting the wound (Eleftherianos & Revenis, 2010).

3.1.3. Cellular immunity

Cellular immune responses in honey bees are mediated by circulating hemocytes. In honey bees, plasmatocytes were identified as the most abundant, followed by granulocytes, oenocytoids, coagulocytes and prohemocytes (Richardson et al., 2018; Gábor et al., 2020; Yelkovan et al., 2021). Hystad et al. (2017) identified mainly granulocytes as phagocytic cells

in honey bees and these cells are likely involved also in the nodulation and encapsulation process as shown in different insect species (Ribeiro & Brehélin, 2006).

Phagocytosis is a conserved mechanism for the rapid elimination of pathogens. The recognition of PAMPs by PRRs activates intracellular signaling cascades resulting in the formation of a phagosome to engulf pathogens by the phagocyte. The phagosome with internalized pathogens fuses with a lysosome and forms a phagolysosome, where the pathogens are degraded (Melcarne et al., 2019). Beyond pathogen elimination, phagocytosis contributes to immune homeostasis and tissue maintenance also by clearing apoptotic bodies and abiotic particles.

While some bacteria are phagocytosed, the recognition of different bacteria triggers different cellular responses, such as nodulation followed by melanization, as shown in mosquitoes (Hillyer et al., 2003). Nodulation is a process involved in the elimination of larger groups of pathogens and foreign particles, while a similar process targeting large particles, such as the eggs of parasitic wasps, is known as encapsulation (Lavine & Strand, 2002). In *Galleria mellonella*, the process begins with the recognition of an intruder followed by the aggregation of granulocytes around it and the attachment and clustering of plasmatocytes (Ratcliffe & Gagen, 1977); however, the exact mechanism of nodule formation in honey bees remains to be elucidated. Enclosed pathogens are then neutralized by cytotoxic substances (Nappi et al., 1995). Nodulation and encapsulation are regulated by eicosanoids, oxygenated metabolites of polyunsaturated fatty acids (Bedick et al., 2001).

Interestingly, cellular immunity in honey bees is influenced by their age and polyethisms. Schmid et al. (2008) showed that the numbers of hemocytes decrease with age in all honey bee castes. In workers, the process is likely mediated by the increase in levels of juvenile hormone that occurs during the transition from nurses to foragers (Amdam et al., 2004). The increase of juvenile hormone reduces levels of vitellogenin, which serves as a crucial zinc donor for hemocytes. Resulting zinc deficiency then triggers pyknosis of hemocytes (Amdam et al., 2004). Consequently, foragers exhibit a reduced rate of phagocytosis (Hystad et al., 2017) and lose the ability to form nodules, whereas nurses maintain nodule formation at levels comparable to those of other insect species, such as *Manduca sexta* (Miller et al., 1994; Bedick et al., 2001). Notably, since long-living winter honey bees retain a similar number of hemocytes like nurses of the summer generation (Dostálková et al., 2021), the immunosenescence in worker bees appears to be driven by polyethic transition rather than chronological age.

3.1.4. Humoral immunity

In insects, humoral immunity refers to defense mechanisms mediated by soluble factors in the hemolymph that neutralize pathogens. These factors are produced by hemocytes, fat body cells, and salivary gland cells. Among the most significant in honey bees are melanin, antimicrobial peptides, lysozymes, and thioester-containing proteins. The production of these compounds is regulated by several immune signaling pathways, which are activated upon the recognition of PAMPs.

One of the constituent components of humoral immunity is the prophenoloxidase cascade. This cascade promotes pathogen recognition and elimination by producing cytotoxic compounds such as melanin and reactive oxygen species (ROS), which are also involved in later stages of encapsulation (Nappi & Christensen, 2005; Binggeli et al., 2014; Dudzic et al., 2015). This cascade involves proteases that convert the inactive enzyme prophenoloxidase (proPO) into its active form, phenoloxidase (PO). Once activated, PO catalyzes a series of biochemical reactions that oxidize tyrosine derivatives into dihydroxyphenylalanine, which is subsequently oxidized along with dopamine into reactive quinones. These quinones then polymerize into melanin, and ROS are simultaneously produced (Söderhäll & Cerenius, 1998). In honey bees, the proPO cascade is constitutively active and likely not subject to immunosenescence (Zufelato et al., 2004).

Other significant effectors in humoral immunity are antimicrobial peptides (AMPs). AMPs are small, positively charged, hydrophobic molecules with the ability to eliminate bacteria, fungi, and viruses (Danihlík et al., 2016). Upon pathogen recognition, AMPs are rapidly synthesized and transported to the site of infection. Their antimicrobial actions include disrupting microbial membrane integrity by inserting into the membrane, interfering with biofilm formation, or targeting intracellular components essential for physiological processes (Zhang et al., 2021). In honey bees, AMPs are usually produced by hemocytes, fat body cells, and salivary glands and secreted into the hemolymph, venom, and royal jelly. Hemolymphic AMPs include defensin (cysteine-rich), apidaecins (proline-rich), abaecin (proline-rich), and hymenoptaecin (glycine-rich). Honey bees produce two defensins: royalisin, which is synthesized in the salivary glands and secreted into royal jelly (Fujiwara et al., 1990), and defensin, which is produced by fat body cells and circulates in the hemolymph (Casteels-Josson et al., 1994). Defensins cause cell lysis of fungi, gram-negative, and gram-positive

bacteria through the disruption of microbial respiration and penetration of cytoplasmic membranes (Ilyasov et al., 2013). Another honey bee AMP, apidaecin, targets mainly gram-negative bacteria (Casteels et al., 1989) through inhibition of translation and chaperone-assisted protein folding (Castle et al., 1999; Zhou et al., 2008). Similarly to apidaecin, abaecin was also shown to bind to bacterial chaperones (Rahnamaeian et al., 2015). Furthermore, abaecin interacts synergistically with hymenoptaecin and perforates bacterial membranes of gram-positive and gram-negative bacteria (Rahnamaeian et al., 2015; Casteels et al., 1993). Royal jelly also contains several AMPs, likely for elimination of the risk of infecting larvae via food. The most significant compounds include the previously mentioned royalisin, which has been shown to affect gram-negative bacteria such as *Paenibacillus larvae* (Bachanová et al., 2002), and jelleines, which affect yeasts as well as gram-negative and gram-positive bacteria (Romanelli et al., 2011). Some bioactive compounds of honey bee venom, such as mellitin, were also proved for their antimicrobial effects against gram-negative and gram-positive bacteria (Fennell et al., 1968).

Lysozymes are the next important bactericidal agents within insect humoral immunity. These enzymes hydrolyze the β-1,4-glycosidic bonds of peptidoglycans in bacterial cell walls (Jolles & Jolles, 1984). Lysozymes act synergistically with AMPs and enhance bacterial clearance (Cytryńska et al., 2001). In honey bees, three lysozyme genes have been identified, two encoding c-type lysozymes and one encoding an i-type lysozyme (Evans et al., 2006).

Another significant group of humoral effectors are thioester-containing proteins (TEPs). These proteins have similar structures to mammalian complement C3 and α2M-F proteins (Blandin & Levashina, 2004). TEPs contain a highly reactive thioester bond, which enables them to work as opsonins for the phagocytosis of yeasts and various bacteria (Levashina et al., 2001; Moita et al., 2005). Additionally, they are likely involved in antiviral defense (Brutscher et al., 2017; Weng et al., 2021). The production of TEPs is regulated by the JAK-STAT signaling pathway (Levashina et al., 2001).

3.1.4.1. Regulation of humoral immunity

The humoral immunity of honey bees is primarily regulated by the Toll pathway, the Immunodeficiency (IMD) pathway and the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway (Evans et al., 2006). While the roles of these pathways have been extensively studied in model organisms such as *Drosophila* and mosquito, their

roles in honey bees were mostly inferred by genomic and transcriptomic studies. Evans et al. (2006) also revealed that honey bees possess only about one third of orthologous immune related genes; however, the immune pathways remain active and functional in honey bees.

Toll pathway

The Toll pathway is an evolutionarily conserved signaling pathway involved in the regulation of immune responses and embryonic development (Belvin & Anderson, 1996). The presence of Gram-positive bacteria and fungi triggers the Toll cascade to produce immune effectors against them (Hetru & Hoffmann, 2009). More specifically, the recognition of these PAMPs by extracellular PRRs activates a proteolytic cascade, leading to the activation of Spätzle, the ligand of the Toll receptor. Two possible Spätzle genes have been identified in honey bees; however, it is unknown which of them binds to the Toll receptor (Evans et al., 2006). The binding of Spätzle to the Toll receptor triggers an intracellular cascade involving MyD88, Tube, and Pelle that results in the degradation of Cactus. All these components were identified in honey bees (Evans et al., 2006). Consequently, Dorsal is activated and translocated to the nucleus, where it regulates the expression of immune effectors (Kidd, 1992; Reichhart et al., 1993). In honey bees, two Dorsal orthologs have been identified (Evans et al., 2006). The Toll pathway controls the transcriptional expression of various immune effectors, such as AMPs and lysozymes, as well as processes controlling cellular responses such as hemocyte proliferation, phagocytosis, and nodulation (De Gregorio, 2002). Furthermore, although not shown in honey bees yet, the Toll pathway can be induced by dsRNA. For instance, Angleró-Rodríguez et al. (2021) demonstrated that a synthetic dsRNA ortholog poly I:C treatment in mosquitos increases the transcription of the Toll6 receptor, downstream effectors defensin A and cecropin C, and results in reduced levels of dengue virus.

Imd and JNK pathway

The IMD pathway is triggered by the recognition of peptidoglycans in cell walls of Gram-negative bacteria (Lemaitre et al., 1995) and is likely conserved between honey bees and *Drosophila* (Evans et al., 2006). In *Drosophila*, the pathway consists of Imd, TAK1, Ird5, Kenny, the Dredd caspase, and the transcription factor Relish (Tzou et al., 2002). Signal transduction within the pathway involves the cleavage of Imd by Dredd, which activates the Tab2/Tak1 complex. This complex phosphorylates the IKK complex, which phosphorylates

Relish. Once activated, Relish translocates to the nucleus and regulates the transcription of immune effectors such as AMPs.

JAK-STAT pathway

The third pathway regulating immune responses is the JAK-STAT pathway, which is evolutionarily conserved and regulates various cellular processes, including development, metabolism, and immunity (Hu et al., 2021). Although orthologs of genes encoding key components of this pathway have been identified in honey bees, their genome lacks an ortholog for Unpaired, the ligand for receptor Domeless (Evans et al., 2006). Therefore, it is unclear how honey bees trigger this pathway. The JAK-STAT pathway is activated by the binding of a ligand to the dimerized Domeless receptor that phosphorylates Hopscotch and the receptor in cytoplasmic regions. This creates docking sites for STAT proteins, which phosphorylate, dimerize, and translocate to the nucleus, where they regulate the expression of target genes (Hu et al., 2021). In *Drosophila*, this pathway controls hemocyte proliferation and differentiation (Zeidler et al., 2000) and regulates the expression of TEPs (Levashina et al., 2001).

3.1.5. Antiviral immunity

The main antiviral response in honey bees is mediated by the RNA interference (RNAi) pathway. However, cellular responses, such as endocytosis, and humoral responses involving AMPs and TEPs likely also contribute to antiviral defense (Brutscher & Flenniken, 2015). RNAi-mediated antiviral defense is triggered by the recognition of dsRNAs in the intracellular matrix. These dsRNAs are either viral genomes or intermediates of viral transcription (Weber et al., 2006). The endoribonuclease Dicer-2 recognizes and cleaves dsRNAs into small interfering RNAs (siRNAs) of approximately 25 nucleotides in length (Hammond et al., 2000). The siRNAs are then loaded into Argonaute (Ago), which forms the pre-RNA-induced silencing complex (pre-RISC) (Hutvagner & Simard, 2008). Using the Dicer-2-associated cofactor R2D2, one siRNA strand is selected as the guide strand based on thermodynamic properties, while the passenger strand is degraded (Ui-Tei et al., 2012). The RISC complex is then assembled with the assistance of various cofactors and heat shock proteins. RISC maturation is completed when the guide siRNA strand is methylated at its 3′ terminal nucleotide. The mature RISC complex then identifies viral sequences and cleaves them using

the catalytic activity of Ago. This cleavage degrades the targeted RNAs and consequently inhibits viral replication (Hutvagner & Simard, 2008; Wilson & Doudna, 2013). While the RNAi pathway is cell-autonomous, systemic antiviral defense requires the spread of RNAi signals to distant tissues, as described in *Drosophila* (Saleh et al., 2009; Niu et al., 2024). Interestingly, Dicer also works as a dsRNA sensor for the production of Vago, a protein with antiviral activity in *Drosophila* and mosquitoes (Deddouche et al., 2008; Paradkar et al., 2014). Although no functional studies of Vago have been conducted in honey bees, Ryabov et al. (2014) demonstrated that its transcriptional activity correlates with DWV levels. However, in bumblebees, Vago expression is linked more to viral virulence than direct antiviral effects (Niu et al., 2016), so its function may differ across insect species. Antiviral defense mechanisms also involve TEPs, which have demonstrated antiviral activity against Dengue and West Nile viruses in mosquitoes (Weng et al., 2021; Cheng et al., 2011). In honey bees, Brutscher et al. (2017) reported an increased expression of Tep7 following infection with Sindbis virus (SINV), and the increased transcription of Tep7 has been linked to increased expression of antiviral protein Bap1 (McMenamin et al., 2021). However, the role of these proteins in honey bees remains largely unexplored.

3.1.6. Trans-generational immune priming

Honey bees also use transgenerational immune priming to enhance their antimicrobial defenses. This form of immunity is maternally induced and mediated by vitellogenin. Vitellogenin produced in the queen's fat body binds components of the bacterial cell wall and is transferred to eggs as a major yolk protein (Salmela et al., 2015). The well characterized example of transgenerational immune priming involves the defense against *P. larvae* infection. Dickel et al. (2022) have shown that when queens were fed heat-inactivated *P. larvae*, their offspring better survived future *P. larvae* challenges. While the transgenerational immune priming provides some protection against bacterial infections, experimental evaluation of its use against viruses showed no significant effects (Leponiemi et al., 2021; Wickramasinghe et al., 2025).

3.2. Social immunity

A honey bee colony is an attractive environment for various pathogens and parasites as it consists of several thousand individuals at different developmental stages cooperation in tight

contact between each other. To prevent their spread, honey bees have evolved a range of protective mechanisms. These defenses include the use of antimicrobial compounds to disinfect food and the colony environment, and behavioral adaptations that limit pathogen and parasite transmission.

One of the honey bee social immunity traits is the collection of propolis. It is a resinous substance, composed of plant secretions collected by honey bees, and it is used as a sealant in the colony. Propolis also serves as material for building protective barriers against larger intruders such as rodents and possesses antimicrobial properties against gram-negative and gram-positive bacteria (Vică et al., 2021), fungi (Jenny et al., 2024), and protozoa (Alenezi et al., 2022). This antimicrobial effect is likely mediated by bioactive compounds such as polyphenols, aromatic acids, and diterpenic acids, but its composition significantly varies across geographic locations (Wagh, 2013), depending on local flora.

Honey bees also produce antiseptic proteins in their hypopharyngeal glands (Hu et al., 2019; Li et al., 2008), including glucose oxidase (GOX) and royalsin, which help control bacterial growth in their food. The GOX catalyzes the conversion of glucose into gluconic acid and hydrogen peroxide, which suppress bacterial growth in larval food and carbohydrate stores (Obeidat et al., 2024; Alshareef et al., 2022; Brudzynski et al., 2011). Interestingly, GOX is produced constitutively, but its expression varies with honey bee density. Individuals reared in smaller groups exhibit higher GOX expression compared to those in larger groups (Jones et al., 2018), suggesting a potential trade-off between immunity and its associated costs. Royalisin promotes bacterial cell lysis (Ilyasov et al., 2013).

Honey bee colonies also use behavioral defenses against pathogens. One such response may involve a colony temperature increase of the whole colony triggered by infection with the parasitic fungus *Ascosphaera apis* (Starks et al., 2000). The temperature increases by approximately 0.6°C, which could potentially inhibit the growth of the fungus and prevent its spread among honey bee larvae (Starks et al., 2000). Experimental *A. apis* inoculation conducted by Goblirsch et al. (2020) confirmed this colony-wide response, but it was not sufficient to eliminate the infection. As a result, it remains unclear whether this temperature rise serves as a direct immune response or is merely a side effect of immune processes, as hypothesized by Goblirsch et al. (2020).

The colony-level prevention of disease spread also stems from the polyethic division of labor among workers of different ages (Simone-Finstrom, 2017). Young bees typically perform

tasks inside the hive, such as feeding larvae and the queen (Johnson, 2009), which reduces the probability of infections caused by external agents. This, in turn, lowers the risk of spreading pathogens within the colony and to vulnerable developmental stages. With increasing age, contact with larvae and the queen decreases, and the oldest workers, which forage for food and are therefore exposed to the external environment, generally do not interact with nurses and larvae (Johnson, 2009).

Another important strategy for controlling disease spread is the exclusion of unhealthy individuals. Rueppell et al. (2010) demonstrated that honey bees treated with CO_2 or hydroxyurea voluntarily left their colony, possibly suggesting an altruistic behavior to protect nestmates when they feel compromised. Nonetheless, honey bees exposed to lipopolysaccharides maintained a degree of social distance and were more likely to be expelled by their nestmates rather than leaving voluntarily (Conroy & Holman, 2022). This behavior could be a contributing factor to honey bee disappearances associated with CCD.

In addition, honey bees can directly detect and remove infected or diseased brood. This trait is referred to as hygienic behavior, and it was shown to reduce the spread of infections caused by *P. larvae, A. apis*, varroa mites, and viruses (Spivak & Reuter, 2001; Gilliam et al., 1983; Medina-Flores et al., 2022; Khan & Ghramh, 2021; Schöning et al., 2012). Honey bee colonies differ in ability to perform hygienic behavior; therefore, it is commonly assessed by tests involving pin killing or freeze-killing of sealed brood (Leclercq et al., 2018). As hygienic are usually considered those colonies that are able to remove > 95% of dead brood within 48 h (Oldroyd, 1996; Spivak & Reuter, 2001). Some honey bees can also remove parasites from their bodies by grooming. A honey bee either removes parasites from itself or manages to remove them from its nestmates (Russo et al., 2020). Both hygienic behavior and grooming play crucial roles in varroa resistance and will be discussed further in the following chapter.

3.3. Varroa resistance

The health and physiology of western honey bees are significantly impaired by the varroa mite, and their survival largely depends on beekeeper interventions. The original host of the varroa mite, *A. cerana*, has evolved several effective strategies to combat varroa mites over millions of years of coevolution, a phenomenon referred to as varroa resistance. After varroa mites spread to Africa, local honey bee populations such as *Apis mellifera scutellata* and *Apis mellifera capensis* were left without beekeeping interventions, that allowed natural selection

to drive the evolving of varroa resistant traits (Allsopp, 2004; Nganso et al., 2018). Hybrids of African honey bees and western honey bees, known as Africanized honey bees, are also varroa resistant (Camazine, 1986). In contrast, western honey bee populations are commonly subjected to constant beekeeper interventions, including selective breeding for honey yield and gentleness, swarm prevention, queen replacement, and regular anti-varroa treatments. These practices inhibit natural selection, thus the ability of managed honey bee populations to adapt to environmental pressures including varroa infestation is limited (Neumann & Blacquière, 2017). However, due to selective breeding programs or natural selection processes, few varroa resistant populations have also emerged within western honey bee populations (Le Conte et al., 2020; Moro et al., 2021). To date, only a limited number of varroa resistant western honey bee populations have been studied in detail. However, it has been shown that varroa resistance is often driven by a combination of complex behavioral and physiological traits, including varroa-sensitive hygiene, recapping of brood cells, and suppression of mite fertility, which frequently act together.

One of the most significant traits contributing to varroa resistance, consistently found across multiple continents in most described varroa-resistant populations, is varroa-sensitive hygiene (VSH). VSH is a specialized form of hygienic behavior in which honey bee workers selectively detect and remove brood infested with varroa mites. Danka et al. (2021) demonstrated that honey bees bred for VSH removed, on average, 44% of varroa infested cells, whereas varroa susceptible honey bees removed only 7%. This trait is likely mediated through olfactory sensing of volatile compounds specifically produced by varroa infested brood. Mondet et al. (2021) identified six compounds produced by infested brood. These are tricosan-2-one, pentacosan-2-one, tetracosyl acetate, heptacosan-2-one, hexacosyl acetate, and nonacosan-2-one. They observed that while these compounds stimulate the olfactory sensilla of all worker honey bees, only varroa resistant honey bees recognized them as signals triggering brood removal. Furthermore, varroa resistant honey bees demonstrated better memory and responsiveness to compounds associated with varroa infested brood in a proboscis extension tests (Mondet et al., 2021; Ivanova & Bienefeld, 2023). Breeding programs for varroa resistance often use VSH as a key marker for the resistance, however, its genetic background is not fully elucidated. Tsuruda et al. (2012) identified two quantitative trait loci associated with VSH located in chromosome 1 and 9, with the regions spanning 63 and 37 genes respectively.

In the context of honey bee defense against the varroa mite, a behavior known as recapping has been also frequently observed (Harris et al., 2012; Villegas & Villa, 2015; Oddie et al., 2021). This behavior involves the opening of sealed brood cells, inspecting these cells, and subsequently resealing them. The exact mechanism by which recapping contributes to the reduction of varroa populations is not fully understood. However, it is speculated that opening infested cells may alter their internal environment, which consequently disrupts the reproductive process of the varroa mite (Kraus & Velthuis, 1997). Although not extensively studied, recapping has been significantly correlated with varroa resistant populations, which exhibit higher efficiency in targeting and performing this behavior compared to susceptible honey bee population (Oddie et al., 2021).

Another significant characteristic of varroa resistant populations is the suppression of mite reproduction (SMR). SMR refers to the infertility or reduced reproductive ability of the varroa mite foundress. SMR has been repeatedly observed in varroa resistant populations from Sweden and France, as these honey bees are able to reduce the reproductive success of the mite up to 50% (Locke & Fries, 2011; Fries et al., 2007; Scaramella et al., 2023). However, a Europe-wide screening of honey bee populations with diverse geographical and genetic origins revealed that, although at lower levels than in selected varroa resistant French honey bees, SMR occurs with varying efficiency among European honey bees (Mondet et al., 2020). The factors underlying SMR are not yet fully understood. Nevertheless, Scaramella et al. (2023) demonstrated that SMR levels in three varroa resistant populations remained unchanged whether the brood was exposed to worker bees or protected by a cage preventing direct worker contact. This suggests that adult worker honey bees do not directly influence SMR. Instead, SMR may arise due to brood derived factors or/and indirectly as a consequence of reduced fertility in insufficiently mated varroa offspring from cells that underwent recapping or from survivors of VSH as hypothesized by Mondet et al. (2020). Three epistatic quantitative loci on chromosomes 4, 7, and 9 were associated with SMR explaining 5.3%, 3.8%, and 8.7% of the phenotypic variance, respectively (Behrens et al., 2011). The trait is heritable and is used as selection marker in breeding programs.

Honey bees also remove phoretic varroa mites from their bodies through grooming behavior. This involves using their legs and mandibles, either to groom themselves or to be groomed by nestmates (Peng et al., 1987; Land & Seeley, 2004). The expression of this trait is commonly assessed by counting dead varroa mites with visible body damage (Nganso et al., 2017). While this behavior significantly contributes to varroa resistance of *A. cerana* (Peng et al., 1987) and

probably of Africanized honey bees (Mondragon et al., 2005; Russo et al., 2022), its role in reducing mite populations in resistant western honey bees remains unclear (Locke & Fries, 2011; Oddie et al., 2017).

3.3.1. Varroa resistant populations of western honey bees

Since varroa resistance is considered a sustainable solution for decreasing the rates of colony losses, considerable efforts have been made to establish stable varroa-resistant western honey bee populations through either selective breeding or natural selection. The following paragraph briefly describes five varroa-resistant populations that have evolved through natural selection or been managed via breeding programs.

3.3.1.1. Gotland (Swedish) honey bee population

The population was established from 150 honey bee colonies of diverse genetic backgrounds located in eight apiaries across Gotland Island (Fries et al., 2003). These colonies were allowed to swarm freely and were subjected only to minimal beekeeping interventions, such as winter supplementary feeding with sugar and swarm management. After three years, more than 80% of the colonies perished. However, by the fifth and sixth years, colony losses stabilized at levels comparable to those recorded in the first year (Fries et al., 2003). Further investigation revealed that these honey bees form smaller colonies, swarm frequently, and have a varroa infestation rate approximately 50% lower than that of susceptible colonies (Locke & Fries, 2011). Additionally, it was shown that these honey bees reduce varroa mite population growth through the SMR mechanism, while their rates of grooming and VSH do not differ from those of susceptible colonies (Locke & Fries, 2011).

3.3.1.2. Norwegian honey bee population

The Norwegian varroa resistant honey bee population originated from Buckfast honey bee colonies that have been left without anti-varroa treatment since 1997 in Østlandet region of Norway (Oddie et al., 2017). Following the last treatment, the population experienced a substantial decline, and the surviving colonies were subsequently propagated (Oddie et al., 2017). These colonies maintain low varroa mite levels and lower varroa foundress fecundity (Oddie et al., 2017), likely due to uncapping mechanisms, which were recorded at high levels

in these bees (Oddie et al., 2021). However, tests for VSH, SMR, and grooming did not show significant differences compared to local varroa susceptible honey bees (Oddie et al., 2017; Scaramella et al, 2023).

3.3.1.3. French honey bee population

This population was established in Avignon and La Manse during the 1990s from 82 honey bee colonies that had survived for several years without anti-varroa treatment across multiple locations in France (Le Conte et al., 2007). The colonies were then managed with minimal beekeeping interventions, including a complete absence of anti-varroa treatment for seven years (Le Conte et al., 2007). During the first five years, these colonies experienced high mortality; however, mortality decreased in the sixth and seventh years despite the lack of treatment. The surviving colonies consistently had lower varroa mite infestations compared to treated colonies, showed no difference in swarming frequency, and produced less honey than treated colonies (Le Conte et al., 2007). Further characterization of varroa resistant traits revealed SMR (Locke et al., 2012; Scaramella et al., 2023), and transcriptional analysis of these bees showed higher expression of olfactory related genes (Navajas et al., 2008).

3.3.1.4. Russian honey bee stock

The Russian honey bee stock originates from a honey bee population located in Primorsky Krai, Russia. Since *A. cerana* naturally occurs in this region, these honey bees were likely exposed to varroa mites for a long time, allowing them a longer adaptation period compared to other *A. mellifera* populations. These honey bees were later introduced to the United States and subjected to tests for varroa resistance and performance under various environmental conditions (Rinderer et al., 2010). These tests shown that Russian honey bees maintain lower levels of varroa mite infestation compared to *A. mellifera ligustica* primarily due to reduced mite reproductive success, lower brood attractiveness to varroa mites, and a high expression of grooming and hygienic behaviors, including VSH (de Guzman et al., 2007; de Guzman et al., 2008; de Guzman et al., 2015; Unger & Guzman-Novoa, 2009). The best performing colonies were selectively propagated to establish a stable, varroa-resistant stock with low susceptibility to other parasites and pathogens while maintaining comparable honey yields like commercially managed *A. mellifera ligustica* (Bourgeois & Rinderer, 2009; Rinderer et al., 2004).

3.3.1.5. Baton Rouge SMR line

This honey bee line was established in Baton Rouge, USA, from honey bees of unknown genetic origin, selected for grooming, hygienic behavior, a short postcapping stage, and a low varroa mite population. Eight colonies were chosen, and queens were artificially inseminated with the semen of single drones. This methodology reduced genetic variability in the selected colonies and allowed for better discrimination of genetic contributions to specific phenotypes (Harbo & Harris, 1999). Ibrahim and Spivak (2006) observed that these honey bees exhibit higher hygienic behavior than Minnesota hygienic honey bees, suggesting that suppressed mite reproduction in these bees is primarily attributed to the high expression of VSH (Harbo & Harris, 2015).

4. Honey bee health promoting interventions from the side of beekeeper

A significant factor influencing the health of honey bee colonies is the beekeeper. Jacques et al. (2017) showed that inexperienced hobbyist beekeepers experience twice the winter colony losses compared to professionals. This suggests that experienced beekeepers are better equipped to recognize the physiological and health status of their colonies and take timely action when needed. Thus, the knowledgeable management and proactive interventions by beekeepers in preventing colony losses are crucial.

4.1. Management

Effective management practices play a crucial role in controlling honey bee pathogens. Healthy colony management includes regular replacement of old combs, maintaining sufficient diet supplies, and prevention of apiary overcrowding. Old wax combs tend to accumulate pesticides, which weaken larvae and increase susceptibility of honey bees to Nosema infections (Ravoet et al., 2015; Wu et al., 2012). Replacing old combs with lighter ones or wax foundations can help to reduce the risk of infections (Meng et al., 2025). Additionally, maintaining a low hive density within an apiary reduces the transmission of diseases, as overcrowded colonies experience higher rates of drifting and robbing, which promote the spread of varroa mites and associated viruses (Seeley & Smith, 2015). Wide hive spacing and distinct hive placement can significantly reduce pathogen transmission and improve colony survival during winter (Dynes et al., 2019).

Furthermore, a balanced diet consisting of carbohydrates, proteins, fats, and essential micronutrients is required for colony development and resilience (Ansaloni et al., 2025). Nutritional requirements vary between developmental stages and castes within the colony. While larvae and queens require a protein-rich diet for growth and egg production, adult honey bees need primarily carbohydrates (Brodschneider & Crailsheim, 2010). A lack of carbohydrates reduces brood production and may lead to starvation that threatens colony survival. For this reason, beekeepers provide alternative carbohydrate sources after honey harvesting. Winter mortality rates do not significantly differ between colonies fed honey, sucrose syrup, high-fructose corn syrup, or invert syrup (Quinlan et al., 2023; Přidal et al., 2023). However, honey bees fed honey and invert syrup have larger fat bodies, suggesting that

these carbohydrate sources are metabolized more efficiently (Quinlan et al., 2023). Unlike sucrose, inverted syrup does not require enzymatic processing by invertase; therefore, its metabolic processing requires less energy and potentially contributes to richer fat body reserves.

Similarly to carbohydrates, insufficient protein intake weakens the colony. Since nurses cannot produce enough larval food, they are forced to cannibalize young larvae to sustain older ones (Crailsheim & Stolberg, 1989; Schmickl & Crailsheim, 2001). Also, insufficient nutrition weakens honey bee immunity, which potentially causes higher vulnerability of honey bees to diseases. For instance, the shortage of dietary proteins reduces the expression of AMPs (Danihlík et al., 2018) and affects the numbers of hemocytes, their metabolic activity, and the composition of hemocyte populations (Szymaś & Jędruszuk, 2003; Alaux et al., 2010). Consequently, honey bees experiencing protein deficiencies are more susceptible to harmful effects of Nosema infection (Tritschler et al., 2017). Therefore, the intake of proteins is important for honey bees, but it can be challenging during extended periods of rain, seasonal shortages of floral resources, or when honey bees are reared in greenhouses. Van der Steen (2007) demonstrated that nutritional supplements can serve as an effective alternative protein source, supporting normal colony development in the absence of natural pollen.

Besides these general practices, beekeepers also commonly target specifically honey bee pests. Since most commercially kept western honey bee colonies are highly susceptible to varroa infestation, typically perishing within three to five years without beekeeper interventions, beekeepers use various strategies to prevent colony losses caused by varroa mites and associated viruses. These include treatments with acaricidal compounds or the use of biotechnical methods limiting the growth of varroa mite population (Brodschneider et al., 2023). The effectiveness and suitability of these approaches depend on colony conditions, the timing within the beekeeping season, and environmental factors such as temperature. The following paragraphs will be devoted to the most used approaches in Europe (Brodschneider et al., 2023).

4.2. Approaches targeting varroa mites

4.2.1. Synthetic acaricides

Acaricidal substances used in apiculture can be either of synthetic or natural origin. The most commonly used synthetic acaricides include pyrethroids, organophosphates, amitraz, and

organic acids. Pyrethroid-based compounds such as fluvalinate, acrinathrin, and flumethrin are typically administered to honey bee colonies through strips impregnated with the active substance, fumigation, or by directly applying the solution onto capped brood. Their acaricidal effect is based on neurotoxic activity. Specifically, pyrethroids prolong the period of voltage-gated sodium channel opening, which increases the influx of sodium into neural cells (Soderlund, 2012). Neurons then cannot generate and transmit action potentials, which is fatal for the targeted organism. Initially, pyrethroids were highly effective, with efficacy reaching approximately 95% (Gregorc & Škerl, 2007). However, their intensive use has led to the spread of resistant strains. González-Cabrera et al. (2013) found that varroa mites resistant to pyrethroids have a substitution of an amino acid in the voltage-gated sodium channels. As a result, a significant decline in pyrethroid efficacy and an increased occurrence of resistant varroa mites have been reported worldwide (Thomson et al., 2002; Morfin et al., 2022; Millán-Leiva et al., 2021). Consequently, these compounds are no longer universally reliable; therefore, many beekeepers shifted to alternative acaricides as the primary method for varroa control. Moreover, pyrethroids are lipophilic and highly soluble in honey bee wax, so their residues accumulate in combs and wax foundations (Tsigouri et al., 2004; Bogdanov et al., 2015). Residues have also been detected in honey (Tsigouri et al., 2004; Bogdanov et al., 2015). In addition, sublethal doses of pyrethroids have serious negative impacts on honey bees, including reduced queen weight (Haarmann et al., 2002), impaired olfactory ability and learning (Ko et al., 2022; Frost et al., 2013), and decreased foraging performance (Wu et al., 2022).

Other widely used groups of acaricides are organophosphates such as coumaphos. These compounds are applied to honey bee colonies using impregnated strips or by directly administering their solution inside the hive. Coumaphos works through its metabolic intermediate corox, which is produced via cytochrome P450 monooxygenases. Corox subsequently disrupts cholinergic signaling by acetylcholinesterase inhibition (Vlogiannitis et al., 2021). Since the clearance of acetylcholine is necessary for the transmission of neural signals, its inhibition leads to dysfunctions of the nervous system and the death of the organism. The efficacy rate of coumaphos reaches up to 96–97% (Zikic et al., 2020). However, resistance to coumaphos was reported in varroa mite populations in the USA (Pettis, 2004; Elzen & Westervelt, 2002) and Argentina (Maggi et al., 2009). This resistance is likely caused by reduced expression of cytochrome P450 monooxygenases in mites that prevents the conversion of coumaphos into its toxic form (Vlogiannitis et al., 2021). In addition, the treatment with coumaphos leaves residues in honey bee products (Kast et al., 2020; Bogdanov

et al., 2015). Furthermore, it was shown to negatively impact antioxidative mechanisms in honey bees (Zikic et al., 2020) and was associated with increased mortality and reduced weight in queens (Pettis et al., 2004).

Amitraz is one of the most commonly used compounds for controlling varroa mites. This substance belongs to the formamidine pesticide family and has a broad acaricidal and insecticidal spectrum (Gupta & Milatovic, 2014). Amitraz works through its binding on octopamine receptors in neurons, which disrupts transmission of neural signals and results in paralysis and death of the organism (Davenport et al., 1985; Gupta & Milatovic, 2014). Its efficiency reaches up to 95% in varroa mites (Semkiw et al., 2013). However, the resistance of the varroa mite to amitraz has spread and been reported in several countries, including Canada, Mexico, Argentina, and France (Bahreini et al., 2025; Rodríguez-Dehaibes et al., 2015; Maggi et al., 2010; Marsky et al., 2024). Resistant varroa mites in Canada were identified to carry a mutation in the Octβ2R gene, likely disabling the binding of the acaricidal molecule on the receptor (Bahreini et al., 2025). In terms of residues, amitraz itself is not detectable in honey bee products, as it rapidly hydrolyzes into 2,4-dimethylaniline through the intermediates N-2,4-dimethylphenyl-N-methylformamidine and N-2,4-dimethylphenylformamide (Jiménez et al., 1997). These toxic metabolites have been detected in honey (Jiménez et al., 1997; Pohorecka et al., 2018). Moreover, amitraz exposure has been associated with physiological effects in honey bees. It has been shown that amitraz increases cardiac activity and reduces the survival of diseased honey bees (O'Neal et al., 2017; Zufriategui et al., 2024). Additionally, when honey bees are exposed to amitraz alongside other pesticides, their learning and memory abilities are impaired (Begna & Jung, 2021).

4.2.2. Alternative acaricidal treatment

Since synthetic acaricides used in beekeeping often leave residues in honey bee products and varroa mites have developed resistance to them, the use of alternative treatments such as organic acids and plant essential oils has increased in Europe (Brodschneider et al., 2023). The main advantage of these compounds is that varroa mites remain sensitive to them (Kosch et al., 2024; Kosch et al., 2025). Contamination is generally not considered an issue as long as the quality of the products remains unchanged. Therefore, the treatments are usually applied when harvestable honey is not present in the colony.

Currently, formic acid, oxalic acid, and thymol are commonly used alternative acaricides against varroa mites (Brodschneider et al., 2023). Formic acid is used in beekeeping due to its ability to target not only phoretic mites, but also mites in capped brood cells (Calis et al., 2015). While its exact mechanism of action is not fully understood, it likely inhibits mitochondrial respiration in mites by oxidizing cytochrome C (Nicholls, 1975). Its effectiveness and potential harm to honey bees depend on careful synchronization with ambient and colony conditions (Underwood & Currie, 2003; Steube et al., 2021). Despite its advantages, formic acid treatments can negatively affect honey bee health. Gregorc et al. (2004) demonstrated that organic acid application induces cell death in honey bee larvae, leading to brood loss (Hendriksma et al., 2024). Additionally, formic acid has been shown to reduce queen survival (Hendriksma et al., 2024) and impair worker bee memory (Gashout et al., 2020).

Dissimilarly to formic acid, oxalic acid only impacts phoretic mites because it does not penetrate brood cells. While the exact mechanism remains still unclear, it has been suggested that oxalic acid crystals may reduce the ability of varroa mites to attach to the honey bee's body (Papežíková et al., 2017). Therefore, oxalic acid should be applied to a broodless colony, which increases its effectiveness up to 98% (Higes et al., 1999; Nanetti et al., 2003). Moreover, treating during broodless periods reduces the risk of brood mortality, as oxalic acid induces cell death in larvae (Gregorc et al., 2004; Hatjina & Haristos, 2015).

Thymol is an essential oil derived from the plant *Thymus vulgaris*, and it probably acts as a neurotoxin, specifically affecting GABA receptors (Bava et al., 2023). Efficacy can reach 95% when applied to broodless colonies (Floris et al., 2004; Kyol & Yeninar, 2016), and it is advantageous to combine thymol application with queen caging (Mahir, 2018). Thymol is a lipophilic substance, so its residues accumulate in wax (Floris et al., 2004). Thymol also reduces olfactory sensing of honey bees, which negatively affects the ability to uncap and eliminate deceased brood (Colin et al., 2019). In addition, thymol is toxic for larvae (Charpentier et al., 2014), resulting in a decrease of the brood in treated colonies (Floris et al., 2004).

Currently, a novel bio-pesticide targeting varroa mites, Norroa™ based on vadescana dsRNA, was approved for use in the USA. Vadescana is designed to mediate the knockdown of the varroa mite's calmodulin, a protein binding intracellular Ca^{2+} and involved in intracellular signal transduction pathways. McGruddy et al. (2024) showed that feeding colonies with this dsRNA significantly decreased the reproductive capabilities of varroa foundresses in brood

cells without any observed negative effects on honey bee pupae. However, anti-varroa control using RNAi has been under experimental testing for a long time. For example, Garbian et al. (2012) showed that feeding honey bees with a mixture of 14 dsRNAs, including sequences of critical cell components such as Varroa α-tubulin, RNA polymerases, ATPases, and apoptosis inhibitors, resulted in a 61% reduction in the varroa population. Interestingly, these sequences were later used in plasmids transferred into the bee gut symbiotic bacterium *Snodgrassella alvi* to produce dsRNAs mediating the knockdown of varroa-specific genes directly in honey bees, resulting in quicker mortality of varroa mites feeding on honey bees with the engineered bacterium (Leonard et al., 2020). The anti-varroa treatment based on RNAi is considered sustainable and eco-friendly, therefore, the administration of this approach in beekeeping practice can likely be expected in the future.

4.2.3. Biotechnical methods

These approaches slow the growth of the varroa population without the use of chemical substances or can be used alongside acaricidal treatments to enhance their efficacy. The most commonly used biotechnical methods include drone brood removal, trapping combs, queen caging, thermotherapy, and screening the health status of colonies (Bubnič et al., 2021).

Since varroa mites preferentially infest drone brood, and the majority of them can be found within capped drone cells (Fuchs, 1990), removing capped drone brood can significantly reduce the varroa population without negative effects on colony strength or honey yield (Calderone, 2005). V arroa population growth can also be reduced through brood comb trapping. This trapping is an empty brood comb, which is inserted into the colony, allowed to be laid and capped, and consequently removed along with the invading varroa mites from the colony (Charrière et al., 2015). Worker brood combs with caged queens can also be used as trappings (Maul et al., 1988). Also, the reproduction of varroa mites can be prevented by induction of a broodless period in the colony using caging of queens. Another approach to varroa control is thermotherapy. This method is based on increasing the colony temperature to levels that are not harmful to honey bees but can kill mites or reduce their fertility (Goras et al., 2018; Le Conte et al., 1990). Thermotherapy uses specialized thermal devices, such as hives designed to increase internal temperatures (Bičík et al., 2016), or thermal boxes in which combs of infested brood are warmed (Porporato et al., 2022).

Additionally, honey bee colony losses can be prevented by monitoring varroa mite populations. Regular monitoring allows for early detection of infestation risks and beekeeping intervention before infested colonies are significantly harmed. The most commonly used methods of monitoring include counting dead varroa mites on bottom boards, performing alcohol or soapy water washes, and inspecting capped brood for mite infestation (Zemene et al., 2015).

Besides these practices generally preventing the spread of varroa mites and pathogens, there are some mostly experimental procedures on how to reduce specific pathogens.

4.3. Approaches targeting other groups of pathogens

4.3.1. Viral infections

Currently, there are only limited options for controlling pathogenic viruses in honey bee colonies. Since some of the most harmful viruses, such as DWV and IAPV, are closely associated with varroa mite infestations, their spread can be prevented through lowering varroa population levels in honey bee colonies. However, the removal of varroa mites does not eradicate viruses from treated honey bee colonies but results in the lowering of viral loads to subclinical levels that still could affect honey bee lifespan and performance (Locke et al., 2017); therefore, there is a need for targeted approaches to control honey bee viruses. Although there is a lack of antiviral treatments in apiculture, some experimental approaches have shown promising results. One such approach involves feeding honey bees with viral dsRNA. Honey bees fed with viral dsRNA during their larval stage were protected against DWV, IAPV, and SBV in adulthood (Desai et al., 2012; Liu et al., 2010; Maori et al., 2009). This suggests that the administration of viral dsRNA induces horizontal immunization, which may be useful for vaccine development in the future. Another potential method for reducing the risk of viral infections involves feeding honey bees with bioactive compounds from certain plants and mushrooms. For example, feeding honey bees with thyme oil, grape pomace, and extracts from polypore mushrooms significantly reduced loads of some viruses (Parekh et al., 2021; Pascual et al., 2023; Stamets et al., 2018). Also, viral loads in honey bees can be decreased using thermotherapy (McMenamin et al., 2020).

4.3.2. Bacterial infections

Pathogenic bacteria are responsible for serious honey bee diseases, such as AFB and EFB. The methods beekeepers use to manage these infections vary significantly between countries. In the USA, beekeepers can treat these infections using antibiotics, whereas most European countries prohibit antibiotic use and require the burning of infected apiaries. However, there are few novel experimental treatments. One of those is a vaccine against AFB, which is based on heat-inactivated *P. larvae* and is fed to the queen to protect her offspring from AFB susceptibility (Dickel et al., 2022). Although this vaccine is not yet available in Europe, it has been approved for use in the USA. Another promising treatment involves the use of probiotics in honey bee food. Truong et al. (2023) demonstrated that larvae fed certain *Lactobacillus* species, which are naturally found in the honey bee gut, showed antimicrobial activity and increased survival when exposed to *P. larvae* infections. These probiotics were also found to have antimicrobial activity against *M. plutonius*. Additionally, plant-derived essential oils reduce the impact of *P. larvae* in vitro (Alonso-Salces et al., 2017).

4.3.3. Microsporidian infections

Levels of *Nosema* can be reduced using the antibiotic fumagillin dicyclohexylamine (Peirson & Pernal, 2024); however, this product is not permitted in many European countries. Another strategy is the application of plant-based treatments, such as powder of the seeds of *Brassica nigra* and *Eruca sativa* (Nanetti et al., 2021), garlic (Kuvancı et al., 2020), and laurel (Porrini et al., 2011). More recently, a novel treatment using dsRNA delivered in a liposomal carrier showed a ten-fold reduction in *N. ceranae* spore levels (Qi et al., 2024).

5. Microbiota of the honey bee

Honey bee microbiome plays an important role in maintaining honey bee health. Honey bees have a relatively simple and stable core microbiota, which was shown to significantly influence their immunity, digestion, and physiology. Additionally, the ability to generate gnotobiotic honey bees allows to precisely study the impact of microbial communities on their hosts. Growing evidence shows the importance of gut microbiota in protecting honey bees against pathogens and various stressors. This chapter explores current knowledge on the honey bee microbiome and its contributions to honey bee health.

5.1. Gut microbiota in honey bee ontogeny

From egg hatch to the senescent phase of a worker honey bee life, the bacterial community undergoes dynamic changes in response to the developmental transitions of the honey bee host. As a holometabolous insect, honey bees progress through distinct stages, including the larval phase, pupal metamorphosis, and emergence as an adult imago. Additionally, adult honey bees exhibit worker polyethism. Each of these life stages and behavioral shifts is accompanied by corresponding changes in the associated microbial community.

Observations of the gut microbiota composition in larval worker honey bees vary across studies. While Martinson et al. (2012) detected no traces of bacterial 16S rRNA genes in most larvae at the 3rd or 5th instar, Vojvodovic et al. (2013), using culture-based methods followed by 16S rRNA sequencing, observed that honey bee larvae were initially colonized by Acetobacteraceae and *Lactobacillus kunkeei*, followed by continuous colonization by *Bifidobacterium*, *Fructobacillus,* and *Lactobacillus* (Firm-4 and Firm-5). Hroncová et al. (2015) also characterized bacterial profiles across different larval instars and found minimal bacterial presence in the L1 instar but observed progressive colonization by *Snodgrassella alvei*, followed by *Gilliamella* and *Lactobacillus* (Firm-4 and Firm-5). Interestingly, although some larvae were completely free of *Lactobacillus*, bacterial taxa of *Frischella perrara*, *Snodgrassela alvei*, and *Gilliamella apicola* were present in the majority of the tested larvae. Nonetheless, bacterial colonization exhibited significant inter-individual and inter-colony variability (Hroncová et al., 2015), suggesting that the colonization process lacks a consistent pattern.

However, inconsistencies in the bacterial colonization of larval honey bees likely do not influence the gut microbiota of adults. Kowalik et al. (2021) shown that larvae fed on different

diets and harboring distinct bacterial communities still develop into adults with typical core gut bacterial profile. This effective decoupling of larval and adult microbiota is likely facilitated during pupal metamorphosis. During this stage, the organism protects itself against septicemia caused by gut bacteria by releasing antibacterial compounds, such as lysozyme and AMPs from the replacement gut epithelium (Russell & Dunn, 1991; Russell & Dunn, 1996; Johnston et al., 2019). The passage from the midgut to the hindgut is closed in larvae, and its opening during the prepupal stage is followed by defecation, through which the prepupa likely loses gut bacteria. As a result, honey bee pupae are typically germ-free or exhibit sharply reduced bacterial abundance (Hroncová et al., 2015; Lanh et al., 2022). Newly emerged honey bees harbor only a small number or no bacteria (Gilliam, 1971; Martinson et al., 2012; Powell et al., 2014; Dong et al., 2020). Adult honey bees subsequently acquire their core gut microbiota within 4 to 6 days through trophallaxis, contact with feces, and interactions with the hive environment (Powell et al., 2014). As honey bees age, their gut microbiota becomes increasingly diverse, especially in the forager stage (Yun et al., 2018) when the honey bee are in the most contact with the external environment.

The gut microbiota of adult worker honey bees is relatively simple at the species level, consisting of approximately five core bacterial species that are consistently present in every honey bee worker, regardless of geographic location (Moran, 2015). These core species include *Snodgrassella alvei*, *Gilliamella apicola*, *Lactobacillus* (Firm-4 and Firm-5), and *Bifidobacterium*. Additionally, *Frischella perara*, *Bartonella apis*, *Bombella apis*, and *Commensalibacter* are often detected in honey bee gut samples, though their presence is not consistent, and they are most likely present in aged honey bees (Moran, 2015; Ellegaard & Engel, 2019). Together, these bacteria account for more than 95% of the bacterial biomass in the honey bee gut. These bacterial species are adapted to colonize specific compartments of the honey bee gut. *Bifidobacterium* and *Lactobacillus* primarily inhabit the rectum, while *Snodgrassella* and *Gilliamella* are found in the midgut and ileum (Callegari et al., 2021). In the ileum, *Snodgrassella* and *Gilliamella* form a biofilm layer, with *Snodgrassella* adhering directly to the gut cuticle, while a thin layer of *Gilliamella* overlays *Snodgrassella* and faces the gut lumen (Martinson et al., 2012). Additionally, Martinson et al. (2021) observed small pockets of *Lactobacillus* Firm-5 along the ileum wall, suggesting that it may also be part of the biofilm. Despite that honey bee gut microbiota is relatively simple at the level of species, each species further diversified into various strains often harboring different metabolic function and implication for honey bee physiology (Ellegaard & Engel, 2019; Powell et al., 2016)

5.2. Honey bee bacteria in health

Honey bee gut microbiota plays an important role in honey bee health via various functions. These microorganisms participate in the digestion of dietary compounds, thus contributing to the availability of nutrients for the functioning of honey bee organisms, as well as participating in the defense against pathogens.

The honey bee gut microbiota predominantly encodes genes for carbohydrate metabolism, suggesting that these microbes are mainly fermenters. They produce various short-chain fatty acids such as acetate, propionate, and butyrate, which serve as nutritional substrates for gut epithelial cells and enhance gut barrier function. Zhang et al. (2017) demonstrated that honey bees with an established microbiota exhibit significantly higher body weight, elevated hemolymph amino acid levels, and increased vitellogenin expression compared to honey bees without gut microbiota. This effect is likely attributed to enhanced sucrose sensitivity driven by elevated insulin signaling (Zheng et al., 201). An important honey bee symbiont from the point of honey bee nutrition is *G. apicola*, because of its ability to break down pectin (Engel et al., 2012). Pectin is a polysaccharide found in the exine of pollen grains, for which honey bees do not produce enzymes to degrade. Therefore, *G. apicola* contributes to the nutrition of honey bees by degrading this polysaccharide into its primary component, galacturonic acid, as shown by Zheng et al., (2017).

Beyond its role in nutrition, the honey bee gut microbiota also plays a crucial role in detoxification. Wu et al. (2020) showed that gut bacteria promote the expression of P450 detoxifying enzymes, which help honey bees to better withstand the harmful effects of pesticides. The disruption of gut microbiota induced by antibiotic treatment resulted in reduced survival of honey bees after their exposure to fluvalinate and thiacloprid.

In addition to enhancing honey bee nutrition and detoxification, gut microbiota also plays an important role in immune priming. Kwong et al. (2017) demonstrated that honey bee gut symbionts stimulate the immune system by inducing the production of apidaecin and hymenoptaecin. The increased production of apidaecin was likely attributed to *S. alvi*, and the levels of this AMP were similar to those observed in honey bees with a complete microbiota. Honey bee symbionts were found to be largely resistant to the antimicrobial effects of apidaecin, whereas endogenous *E. coli* was highly susceptible. This suggests that microbiota induced apidaecin production helps to combat bacterial invaders without harming beneficial

symbionts. Supporting this, honey bees with an intact microbiota had increased survival and enhanced *E. coli* clearance upon bacterial challenge compared to controls lacking normal microbiota (Kwong et al., 2017). Moreover, *S. alvi*-induced immune priming led to improved honey bee survival after *Serratia* infection and more effective clearance of this pathogen (Horak et al., 2020).

Furthermore, Lang et al. (2022) demonstrated that specific strains of *Lactobacillus* and *Gilliamella* enhance the clearance of the honey bee bacterial pathogen *Hafnia alvei*. Honey bees monocolonized with certain strains of these bacteria exhibited improved resistance against *H. alvei*, whereas other strains of the same species, or different gut microbiota members such as *Bartonella* or *Bifidobacterium*, did not significantly affect levels of *H. alvei*. *Lactobacillus* strains associated with enhanced protection against *H. alvei* induced higher expression of abaecin, hymenoptaecin, and lysozyme. Conversely, antibiotic-treated honey bees had significantly reduced survival after the *H. alvei* infection (Lang et al., 2022).

Frischella perrara also strongly induces immune responses in honey bees. Its presence is associated with the formation of a melanized scab in the ileum and the upregulation of several immune genes, including those involved in the proPO cascade, PPRs, and AMPs (Emery et al., 2017). Additionally, *F. perara* triggers a stronger immune response compared to *S. alvi* (Emery et al., 2017). However, its overall impact on honey bee health remains controversial, as products of the colibactin pathway in *F. perara* have a cytotoxic effect (Engel et al., 2015).

The gut microbiota also inhibits the growth of non-core bacterial species. Raymann et al. (2017) demonstrated that antibiotic-induced dysbiosis in the honey bee gut promoted an increased abundance of *Halomonadaceae*, *Serratia*, and certain fungal species. Long-term antibiotic exposure resulted in persistent microbial imbalances, even after re-exposure to hive associated bacteria. Consequently, antibiotic treated honey bees exhibited reduced survival after *Serratia* infection (Raymann et al., 2017). Moreover, dysbiotic honeybees were more susceptible to *L. passim* infection (Schwarz et al., 2016).

Beyond its effects on bacterial and protozoan infections, honey bee gut microbiota may also influence viral infections. Dosch et al. (2021) found that honey bees lacking an established microbiota had significantly reduced survival after DWV infection compared to those with a normal microbiota. However, studies on the role of gut microbiota in viral infection tolerance and reduction remain limited, and the mechanisms underlying this protection have yet to be fully elucidated.

OBJECTIVES OF THE THESIS

The overarching aim of this thesis was to explore factors affecting honey bee health from different perspectives, with a particular focus on honey bee viruses, gut microbial communities associated with varroa-resistant colonies, and properties of wax cappings that support normal bee development and allow the passage of substances involved in hygienic behaviour.

Most honey bee colonies managed in Europe and North America are highly susceptible to viral infections associated with varroa mites. Strategies to reduce these infections are limited and largely preventative. Based on our preliminary data, we hypothesized that alcohol extracts of some plants and mushrooms may have antiviral properties against honey bee viruses. Among those preliminarily tested, the alcohol extract of the gypsy mushroom (*Cortinarius caperatus*) appeared especially promising. Therefore, the aims of Chapter I were (1) to test the antiviral effect of gypsy mushroom extract against DWV in honey bees using cage experiments, (2) to assess its impact on honey bee lifespan, (3) to validate the findings of cage experiments through winter supplementary feeding in hives, and (4) to assess whether mushroom residues are present in honey.

Despite extensive research on varroa-resistant honey bee populations, important gaps remain in our understanding of how they differ from varroa-susceptible colonies. We hypothesized that gut microbial communities may also differ between these two honey bee strains. The aim of Chapter II was to examine the gut bacterial communities of varroa-resistant Gotland honey bees compared with non-resistant bees managed at the shared apiary. Using an existing 16S rRNA dataset generated by Thaduri et al. (2021), we aimed (1) to construct co-occurrence networks of the gut bacteria and analyze their similarities and differences, and (2) to determine bacterial correlations with five honey bee pathogenic viruses.

Integral parts of each honey bee nest are the wax combs, which mainly serve to store dietary supplies and provide sites for brood development. The functions of cappings of the two types of combs differ significantly. While the cappings of storage combs protect honey against humidity and fermentation, the cappings of brood cells must remain permeable to gases and water to allow developing pupae to breathe. Although these two types of cappings must differ in important characteristics, little is known about their ultrastructure and chemical and physical properties. For this reason, the main aims of Chapter III were (1) to assess differences in the chemical and structural characteristics of the two types of cappings; (2) to measure their CO_2 conductance; and (3) to quantify pupal respiration and assess the resulting CO_2 gradients

across the wax cappings. Addressing these unknowns is essential for our further research testing the hypothesis that changes in pupal respiration caused by parasites and/or pathogens (including viral infections) could potentially serve as a signal contributing to the induction of hygienic behavior in worker bees.

CHAPTER I: Alcohol extract of the gypsy mushroom (*Cortinarius caperatus*) inhibits the development of Deformed wing virus infection in western honey bee (*Apis mellifera*)

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Alcohol extract of the gypsy mushroom (*Cortinarius caperatus*) inhibits the development of Deformed wing virus infection in western honey bee (*Apis mellifera*)

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ABSTRACT

Deformed wing virus (DWV) transmitted by the parasitic mite *Varroa destructor* is one of the most significant factors contributing to massive losses of managed colonies of western honey bee (*Apis mellifera*) subspecies of European origin reported worldwide in recent decades. Despite this fact, no antiviral treatment against honey bee viruses is currently available for practical applications and the level of viral infection can only be controlled indirectly by reducing the number of *Varroa* mites in honey bee colonies. In this study, we investigated the antiviral potential of the gypsy mushroom (*Cortinarius caperatus)* to reduce DWV infection in honey bees. Our results indicate that the alcohol extract of *C. caperatus* prevented the development of DWV infection in cage experiments as well as after direct application to honey bee colonies in a field experiment. The applied doses did not shorten the lifespan of honey bees. The reduced levels of DWV in *C. caperatus*-treated honey bees in cage experiments were accompanied by significant changes in the gene expression of Tep7, Bap1, and Vago. The *C. caperatus* treatment was not effective against the trypanosomatid *Lotmaria passim*. No residues of *C.caperatus* were found in honey harvested in the spring from colonies supplemented with the mushroom extract for their winter feeding. These findings suggest that *C. caperatus* alcohol extract could be a potential natural remedy to treat DWV infection in honey bees.

1. Introduction

The western honey bee (*Apis mellifera*) is one of the most important pollinators of agricultural crops (Khalifa et al., 2021) as well as wild floral species (Hung et al., 2018). The honey bees pollination service is therefore highly valuable, both economically and ecologically. However, during the last few decades, massive losses have been reported worldwide for managed colonies derived from *Apis mellifera* subspecies of European origin (Neumann & Carreck, 2015). These colony losses are caused by multiple factors including an extensive way of agriculture, insufficient food supply during critical parts of the season, and the spread of parasites and diseases (Hristov et al., 2020; Insolia et al., 2022). The parasitic mite *Varroa destructor* along with associated viruses have been identified as the strong driver of colony losses (Guzmán-Novoa et al., 2010; Wilfert et al., 2016). Also gut pathogens such as the microsporidium *Nosema ceranae* and the protozoa *Lotmaria passim* may harm honey bee health (Marín-García et al., 2022; Gómez-Moracho et al., 2020), contributing to the death of colonies (Ravoet et al., 2013).

Honey bee viruses, particularly Deformed wing virus (DWV), have been shown to be strongly associated with colony losses (Highfield et al., 2009). DWV is a non-enveloped positive single-strand (ss+) RNA virus of the *Iflaviridae* family (Lanzi et al., 2006) that can replicate in honey bee brood as well as in adult tissues (Gusachenko et al., 2020). As the *Varroa* mite serves as an efficient vector for DWV transmission, the virus is the most widespread honey bee virus in *Varroa*-infested colonies, severely affecting honey bee health (Ryabov et al., 2014). Although honey bees have adopted several antiviral defense strategies, including cellular and humoral immune mechanisms (Brutscher et al., 2015; Feng et al., 2020), *Varroa* mite feeding modifies the host immune system, allowing rapid viral replication (Yang & Cox-Foster, 2005; Nazzi et al., 2012; Kunc et al., 2023). This usually results in the manifestation of clinical symptoms in honey bees that have emerged from *Varroa*-infested brood, such

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as wing and abdomen deformities, impaired cognitive functions, shortened lifespan and death (Lanzi et al., 2006).

Beekeepers prevent the development of DWV infection indirectly by reducing the *Varroa* mite vector population. According to our knowledge, no commercially available antiviral treatment for honey bees is currently available on the market. However, a few experimental approaches to cope with honey bee viruses have been reported in recent years. The first involves the addition of dsRNA into honey bee royal jelly to stimulate activation of the RNAi pathway (Desai et al., 2012). This strategy, however, might not always be effective (Yang et al., 2018) and it can also have unintended consequences, such as off-target effects (Nunes et al., 2013). Reduced viral titers have also been observed after exposing bees to a heat-shock regime (McMenamin et al., 2020). Another approach to reduce honey bee viruses is by feeding honey bees with natural supplements that have been shown to possess antiviral properties in other biological systems. For example, honey bees fed a diet supplemented with thyme oil, a commonly used anti-varroa substance, showed enhanced activity of their immune system and reduced DWV loads (Parekh et al., 2021). A similar effect was shown in honey bees after feeding the grape pomace powder-supplemented diet (Pascual et al., 2023). Also, feeding honey bees with extracts from polypore mushrooms *Ganoderma lucidum* and *Fomes fomentarius* significantly reduced the levels of DWV and LSV viruses (Stamets et al., 2018). The antiviral potential of mushrooms was further confirmed by the observation that DWV levels in honey bees were significantly reduced by feeding ß-glucans (Felicioli et al., 2020), common secondary metabolites present in the cell walls of mushrooms.

Secondary metabolites from higher fungi have consistently been demonstrated to be highly potent antiviral agents. Antiviral properties have been described mainly through the actions of polysaccharides, proteins, peptides, polyphenols and triterpenoids. These substances can suppress viral entrance (Pan et al., 2013), replication (Xu et al., 2012), the activity of viral enzymes (El-Mekkawy et al., 1998), cellular and viral protein production (Zhao et al., 2016; Okamoto et al., 2004), and boost host immunity (Wang et al., 2014) against a wide spectrum of viruses, including non-enveloped ss+ RNA viruses (Seo & Choi, 2021). For instance, a polysaccharide from *Agaricus brasiliensis* inhibited Poliovirus (Faccin et al., 2007), and a polysaccharide from *Grifola frondosa* suppressed the expression of the capsid protein VP-1 of Enterovirus 71 (Zhao et al., 2016). However, the underlying mechanisms of fungal antiviral actions are much better understood for enveloped viruses. For example, polyphenols from *Phellinus linteus* and *Glaziella splendens* have an inhibitory effect on neuraminidase of Influenza A (Hwang et al., 2018; Kim et al., 2019), triterpenes from *Ganoderma lucidum* inhibit HIV-1 protease (El-Mekkawy et al., 1998) and lactase isolated from *Lentinus tigrinus* inhibits reverse transcriptase of HIV (Xu et al., 2012). Strong antiviral properties were also reported for *Cortinarius caperatus*, as the protein RC28 isolated from this fungus was shown to inhibit HSV-1 and 2, varicella-zoster virus, influenza A virus, and respiratory syncytial virus (Yan et al., 2015; Piraino & Brandt, 1999).

The objective of this study was to evaluate the potential of *Cortinarius caperatus* as the treatment against viral infection of honey bees. Thus, we examine the ability of the alcohol extract and the dry powder of the mushroom to reduce DWV titers in cage experiments as well as in field experiments using honey bee colonies in hives. Supplementary feeding of the mushroom alcohol extract was also tested for its effect on honey bee lifespan. Moreover, possible presence of mushroom residues in honey was evaluated in field experiments. Our results demonstrated that the alcohol extract of *C. caperatus* can be used as a safe and effective treatment to reduce DWV infection.

2. Methods

2.1. Preparation of mushroom products

Young mushrooms of *C. caperatus* were collected in two places in South Bohemia (Ločenice and Chvalšiny) in September 2020. The fresh mushrooms were cleaned of any raw impurities, thinly sliced, and separated into two batches. The first batch of mushrooms was dried in a fruit dryer (24 h at 50 °C) and ground into a fine powder, while the second batch was used for the preparation of alcohol extract. For the preparation of the alcohol extract, 500 g of fresh mushrooms were combined with 1 liter of 70% ethanol and macerated for 14 days at room temperature in the dark. The alcohol extract was then strained through a kitchen sieve with aproximately 0.5 mm pores and kept in the dark at 4 °C.

2.2. Characterization of the experimental honey bee colonies

The experimental apiary is located in the dendrological garden of the Biology Centre AS CR, Ceske Budejovice, Czech Republic (48°58′31.924″N, 14°26′44.671″E; 390 m MSL). Honey bees used in the experiments originated from strong colonies with 2-years-old laying queens of *Apis mellifera carnica*. The colonies used for the field experiments received oxalic acid treatment in winter 2020 and no other acaricides until the end of the experiments in September 2021. Other colonies served as sources of honey bees for the cage experiments. These colonies were managed according to standard practice, that included anti-varroa treatment (amitraz fumigation in autumn, oxalic acid drops in winter, formic acid in summer), honey harvest in June, and sugar syrup feeding in August. The levels of *Varroa destructor* were regularly monitored by alcohol washes and did not exceed 2% level throughout the season.

2.3. Experimental design

Experiments in this study were performed according to the scheme shown in Fig. 1. Briefly, cage experiments were conducted to evaluate the capacity of *C. caperatus* to affect the levels of DWV and *L. passim*. The experiments involved feeding honey bees with sugar syrup supplemented with mushroom extract, as well as feeding dry mushroom powder. Honey bees fed with mushroom extract were analyzed for the expression of immune genes and their lifespan was also assessed. Sugar syrup with mushroom extract was then applied to hives to test its ability to reduce the levels of DWV *in vivo*. Immune gene expression and *Varroa* infestation levels were also determined in the field experiment.

2.4. Supplementary feeding of C. caperatus extract to honey bees in cages

Brood frames containing hatching bees from individual hives in the experimental apiary were transferred to an incubator (35 °C, 70% relative humidity). After 24 h, newly hatched honey bees were briefly anesthetized with CO_2 and then moved to experimental cages (50 honey bees per cage, 6 cages per biological replicate) and kept in an incubator (35 °C, 70% relative humidity). A sample of 25 honey bees was frozen at −80 °C for later examination at 'time 0'. The caged honey bees had *ad libitum* access to drinking water and sugar syrup (1:1) mixed with either 1% or 0.1% alcohol mushroom extract of *C. caperatus*. Honey bees in the control cages were fed sugar syrup with 0.5% pure alcohol instead of mushroom alcohol extract. Every other day, dead individuals were removed, and honey bees received fresh water and sugar syrup. Alternatively, caged honey bees had unlimited access to water and honey containing 1% or 0.1% of dry mushroom powder. Control honey bees were fed pure honey, and these honey bees were given fresh water and a honey diet daily. After 14 days, honey bees from all cages were frozen at −80 °C and stored until further processing.

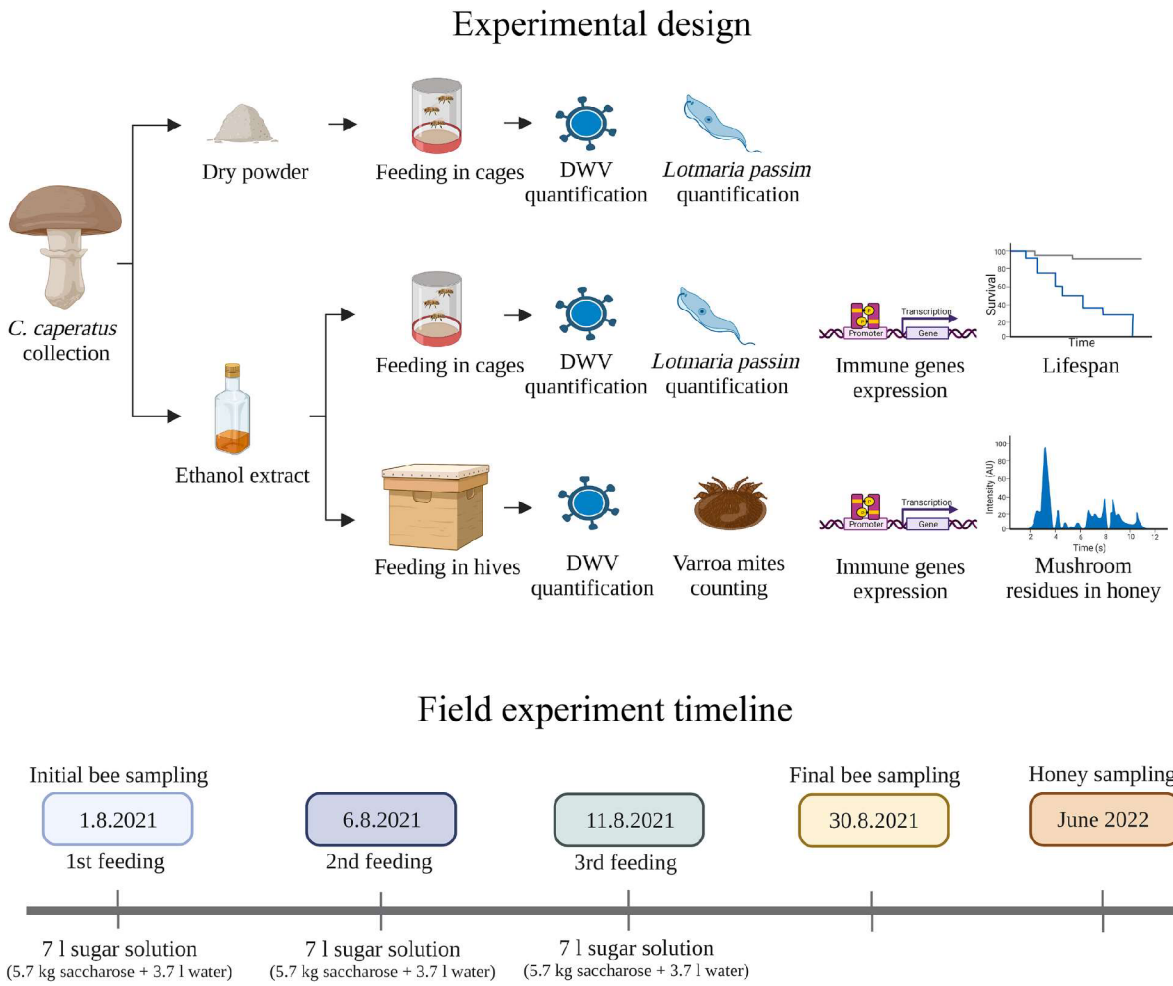

Fig. 1. Experimental design of cage experiments and *in vivo* experiments in honey bee colonies. In cage experiments, the impact of dry *C. caperatus* powder and of *C. caperatus* alcohol extract was examined. While the dry mushroom powder was tested for its effect on the level of honey bee pathogens, the mushroom extract was further examined for its effect on the lifespan of honey bees and on the expression of immune genes . In the field experiment, colonies were fed sugar solution with *C. caperatus* extract according to the time line bellow and honey bees were examined for the levels of DWV, levels of *Varroa* infestation and changes in the expression of immune genes. Additionally, honey from the spring harvest of experimental colonies was tested for the presence of mushroom residues. This scheme was created using BioRender (Agreement number: PX25LG613M).

2.5. Lifespan determination

Lifespan experiments were conducted with three experimental groups of caged honey bees (caging was performed in the same manner as for the supplementary feeding of *C. caperatus*). Each group received a different diet containing either 1% mushroom extract in sugar syrup, 1% mushroom extract in sugar paste, or 1% dry mushroom in a dry pollen substitute. Control groups were fed the same diet without mushroom supplements. All groups had *ad libitum* access to fresh water. Every other day, all groups were provided with fresh food, and dead individuals were removed and counted. The experiments ran until the death of the last honey bee in each group.

2.6. Supplementary feeding C.caperatus extract to honey bee colonies in field experiments

Field experiments were performed at the experimental apiary during the summer of 2021 with colonies of similar strength (honey bees occupying 2 supers, each with 10 frames of 39×24 cm). To design the experimental and control groups as similarly as possible, all colonies were screened for the levels of DWV and evenly distributed into the two groups before the start of the experiment. The groups consisted of 8 (experimental group) and 10 colonies (control group). Samples of 25 honey bees were collected from each colony one day before the feeding and frozen at −80 °C for later examination as time 0. Colonies were fed a total of 21 liters of sugar syrup (17 kg of saccharose: 11 liters of water) in 3 feeding doses at 5-day intervals starting on August 1st, 2021 (Fig. 1). The sugar syrup for experimental colonies contained 1% mushroom extract, while the syrup for control colonies did not contain any additives. After 30 days, samples of 25 honey bees from each colony were collected and frozen at −80 °C until further examination. The level of *Varroa* infestation was assessed in all colonies after amitraz fumigation of broodless colonies at the end of September.

2.7. Homogenization of honey bee samples

For the isolation of nucleic acids, 25 honey bees from each sample were grouped, their heads removed and homogenized (MM300 TissueLyser Mill Mixer, Retch, Haan, Germany) for 3 min. at 30 Hz under liquid nitrogen cooling. Honey bee homogenate was diluted with 1 ml of ice-cold Milli-Q water and used for nucleic acids extraction.

2.8. RNA extraction and cDNA synthesis

Total RNA was isolated from 100 µl of honey bee homogenate using TriReagent (Sigma, St. Louis, MO, USA), precipitated and washed with 70% ethanol according to the manufacturer's protocol. The pelleted RNA was dissolved in 50 µl of nuclease-free water, quantified, and checked for purity using a NanoDrop OneC UV–Vis Spectrophotometer (Thermo Scientific, Waltham, MA, USA). The integrity of the RNA was verified by gel electrophoresis. Complementary DNA (cDNA) was produced from 1 µg of total RNA using a master mix containing 200 U of M−MLV reverse transcriptase (Promega, Madison, WI, USA), 0.4 µg of random primers (Qiagen, Hilden, Germany), 20 µmol of dNTP mix (Sigma, St. Louis, MO, USA), and 20 U of Recombinant RNAsin ribonuclease inhibitor (Promega, Madison, WI, USA) in a total volume of 20 µl. The cDNA synthesis was carried out at 37 °C for 1 h, followed by 15 min. at 70 °C.

2.9. DNA extraction

Genomic DNA was isolated by mixing 100 µl of honey bee homogenate with 150 µl of H-buffer (0.02 M Hepes, 0.05 M KCl, 0.001 M MgSO4, 0.01 M NaCl, 0.005 M NaHCO3, 0.001 M NaH2PO4·2H2O; pH = 7.0), 100 µg proteinase K, and 0.65% SDS (w/v), and incubating the mixture overnight at 50 °C. The resulting mixture was then diluted with 100 µl of nuclease-free water, and DNA was extracted twice using UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v) (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol, followed by alcohol precipitation and washing. The pelleted DNA was mixed with 100 µl of DNase-free water containing 1 ng of RNase A, and dissolved at 68 °C. For quantification by PCR, the DNA was diluted 10-fold in nuclease-free water.

2.10. Quantitative real-time PCR

Quantitative PCR was performed using 10 µl reactions containing 1 × GoTaq® qPCR master mix (Promega, Madison, WI, USA), 3.3 µM forward primer, 3.3 µM reverse primer, and 0.5 µl cDNA reaction or 10 times diluted gDNA. Primers used in this study are listed in Supplementary Table 1. Reactions were carried out in 96-well optical PCR plates in a CFX96 Real-Time System (Bio-Rad, Herkules, CA, USA). Each run included a non-template control. Runs for quantifying pathogens also included 5 samples of plasmid standards spanning dilutions of 5 orders of magnitude, from which a calibration curve was constructed. The thermal cycling program consisted of enzyme activation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 57 °C for 15 s, and elongation at 72 °C for 15 s. Melting curve analysis was performed at the end of each run.

The quantification of pathogens was determined from the cycle threshold (C_t) values of experimental samples and the cycle threshold values of plasmid standard samples containing a known number of target copies. The number of copies of pathogen RNA or DNA was normalized to honey bee housekeeping gene copy numbers (DWV was normalized to Rp 49 expression, *N. ceranae* and *L. passim* were normalized to Actin genomic copy number). Relative levels of pathogens were then calculated as ratios between the normalized pathogen values obtained from experimental or control samples and the normalized pathogen values obtained at time 0.

Relative expressions of immune genes in caged honey bees were calculated according to Vandesompele et al. (2002), with Rp 49 and Actin as reference genes. Relative expressions of immune genes in honey bees from field experiments were calculated using the 2(-ΔΔCT) method (Livak & Schmittgen, 2001) with Rp 49 as the normalization gene.

2.11. Generation of plasmid standards

Plasmids used for calibration curves in the quantitative real-time PCR of *N. ceranae*, *L. passim*, Rp 49, and Actin contained PCR amplicons generated with detection primers (listed in Supplementary Table 1) and cloned into the pGEM®-T Easy vector (Promega, Madison, WI, USA). The inserts were verified by sequencing. Purified plasmids were diluted in water to a concentration of 5 µg per 1 µl and stored at −80 °C until further use.

The plasmid used for the quantification of DWV was created and published by Bradford et al. (2017), and kindly provided to us upon request to the corresponding author.

2.12. Gas chromatography and mass spectrometry

Honey samples were dissolved in distilled water to obtain 1% solution (10 mg/ml). 50 µl of this solution was evaporated using dry nitrogen stream, silylated (50 µl BSTFA + 100 µl pyridine, 2 h at 80 °C) and n-hexane was added to the final volume 1 ml at the end. Samples were analyzed on a gas chromatograph (Trace 1310, Thermo, Bremen, Germany). A Restek Rxi-5MS-Sil column (30 m x 0.25 mm x 0.25 µm film thickness) was used with the flow rate of 1.5 ml min^{-1} of helium as carrier gas. The injection (at 300 °C) was splitless for 1.5 min, then split flow at 100 ml min^{-1} for another 1 min and 5 ml min^{-1} for the rest of the time (gas saver). The oven temperature program was set to 50 °C during injection and for the following 2 min, then increased with a gradient of 40 °C min^{-1} to 200 °C, then at 4 °C min^{-1} to 310 °C, and was isothermal at 310 °C for the rest of the analysis (c. 55 min in total). The eluting compounds were oxidized to CO_2 via IsoLink II interphase (Thermo, Bremen, Germany) at 1000 °C and introduced into a continuous flow isotope ratio MS (Delta V Advantage, Thermo, Bremen, Germany).

2.13. Statistical analysis

Statistical analyses of group comparisons were performed in GraphPad 8 Prism (GraphPad Software Inc., San Diego, CA, USA). A Student T-test was performed when the assumption of normal distribution was met, otherwise, a non-parametric Mann-Whitney test was used. Significance was defined as $p < 0.001$ (***), $0.001 < p < 0.01$(**), $0.01 < p < 0.05$ (*). Lifespan analysis was performed using the 'survival' package (Therneau and Grambsch, 2000) implemented in R studio (R studio team, 2020).

3. Results

3.1. The effect of C. caperatus on DWV viral load and L. passim infection in cage experiments

To investigate whether *C. caperatus* mushrooms affect DWV infection in honey bees, caged honey bees were fed mushroom extract in sugar syrup or dry mushroom powder mixed with honey. Feeding with both forms of mushroom significantly reduced DWV levels in honey bees in a dose-dependent manner (Fig. 2 and Fig. S1). While feeding 0.1% mushroom extract did not affect DWV levels in the test group of honey bees compared to the control group, the viral titer was significantly lower in the group fed with 1% mushroom extract ($p = 0.0004$) (Fig. 2A). Moreover, the DWV levels in honey bees fed with 1% mushroom extract were nearly identical to the initial DWV level detected at the start of the experiment (Time 0), suggesting that the mushroom treatment inhibited the development of viral infection. Feeding 0.1% dry mushroom powder in honey also resulted in a significant decrease in DWV levels, with a difference of one order of magnitude compared to the control group fed with pure honey ($p = 0.0043$) (Fig. 2B). On the other hand, feeding honey containing 1% dry mushroom powder was accompanied by considerably premature mortality of honey bees (data not shown).

We also extended our investigation of *C. caperatus* inhibiting potential to *Nosema ceranae* and *Lotmaria passim*. While *N. ceranae* was not detected in any of the tested samples, *L. passim* was omnipresent.

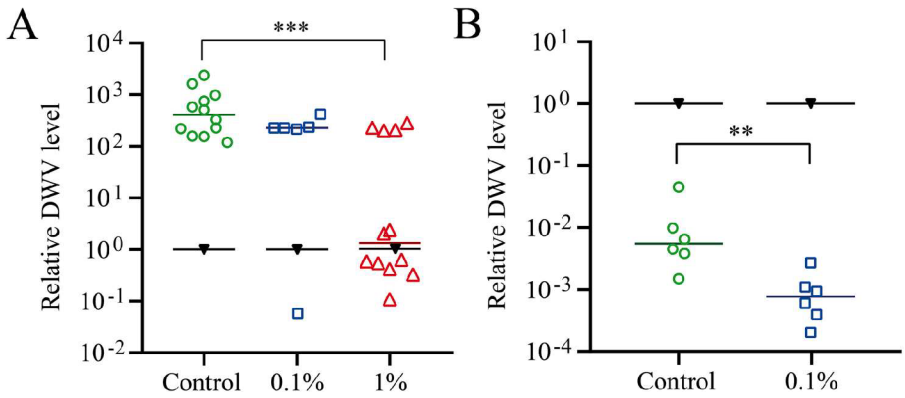

Fig. 2. *Cortinarius caperatus* prevented the progression of DWV infection in caged honey bees. (A) Honey bees fed with 1% mushroom alcohol extract in sugar syrup had significantly lower titers of DWV compared to controls ($p = 0.0004$). The DWV levels of this group were similar to DWV levels at time 0 (black triangle), suggesting inhibition of the development of DWV infection. Red and green horizontal lines represent medians of the honey bee groups, whereas black horizontal lines depict the normalizing value 1. (B) Feeding honeybees with 0.1% dry mushroom in honey significantly reduced DWV levels. Values in the graphs are expressed as ratios of normalized values for individual honey bee samples to the normalized value at time 0. Horizontal lines represent medians of the honey bee groups. Groups of samples are distinguished by the colors and shapes of corresponding marks (black triangle for time 0, green circle for control group, blue square for group treated with 0.1% mushroom and red triangle for group treated with 1% mushroom). Testing of mushroom extract was performed in 2 independent biological replicates (group fed 0.1% extract was not included in the second biological replicate), while the experiment with honey feeding contained a single biological replicate. Statistical analysis was performed using Mann-Whitney *U* test.

However, experimental groups did not show differences in *L. passim* titers compared to controls, neither when honey bees were fed 1% mushroom in sugar syrup (Fig. 3A) nor 0.1% dry mushroom powder in honey (Fig. 3B). These results indicate that *C. caperatus* in the form of alcohol extract or dry powder does not alter levels of *L. passim* in honey bees.

3.2. The effect of *C. caperatus* extract on the expression of the immune genes and on lifespan of honey bees in cage experiment

To better understand the antiviral action of *C. caperatus* extract on honey bees, we quantified the mRNA levels of selected immune genes previously described to be involved in antiviral immune activities (McMenamin et al., 2018; McMenamin et al., 2021). The expression of the majority of selected immune genes, namely Abaecin, Apidaecin, Cytochrome P450 6AS5 (Cyp6AS5), Defensin, Dicer 2 (Dcr2), Glucose oxidase (Gox), and Prophenoloxidase (PPO), was not statistically different between the mushroom-treated and control groups (Fig. 4). However, the expression of genes encoding Thioester containing protein 7 (Tep7) ($p = 0.0300$), and Bee antiviral protein 1 (Bap1) ($p = 0.0241$) was significantly upregulated and the expression of the Vago gene was significantly downregulated ($p = 0.0010$) in the mushroom-treated group. Noticeably, transcriptional changes appeared only in the honey bee group fed with sugar syrup containing 1% mushroom alcohol extract..

To verify the safety of 1% *C. caperatus* alcohol extract for honey bees, we analyzed the lifespan of caged honey bees fed with mushroom extract administered by three different carriers: sugar syrup, sugar paste, and dry pollen substitute. The treatment of 1% mushroom extract did not shorten the lifespan in any of the tested groups (Fig. 5). The group fed with mushroom extract mixed with sugar paste had even a slightly longer survival time at 50% mortality than the control group ($p = 0.0050$) (Fig. 5B).

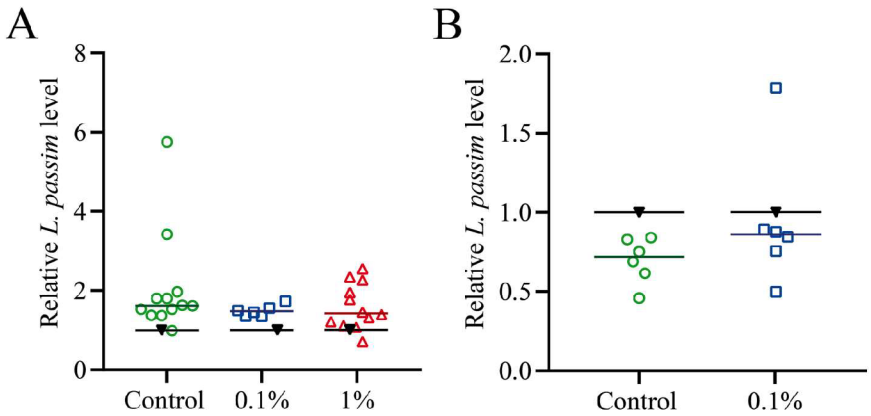

Fig. 3. *Cortinarius caperatus* had no effect on the levels of *Lotmaria passim* in caged honey bees. (A) Feeding sugar syrup supplemented with 0.1% and 1% of mushroom extract and (B) feeding of 0.1% dry mushroom powder in honey did not affect *L. passim* titers in caged honey bees. The graphs display ratios of normalized values for individual honey bee samples to normalized value at time 0. Groups of samples are distinguished by colors and shapes of corresponding marks (black triangle for time 0 green circle forcontrol group, blue square for group treated with 0.1% mushroom, and red triangle for group treated with 1% mushroom). Red and green horizontal lines represent medians of the honey bee groups, whereas black horizontal lines depict the normalizing value 1. Testing of mushroom extract in sugar syrup was performed in 2 independent biological replicates (group fed with 0.1% extract was not included in the second biological replicate), while the experiment with mushroom powder in honey contained a single biological replicate. Statistical analysis was performed using Mann-Whitney *U* test.

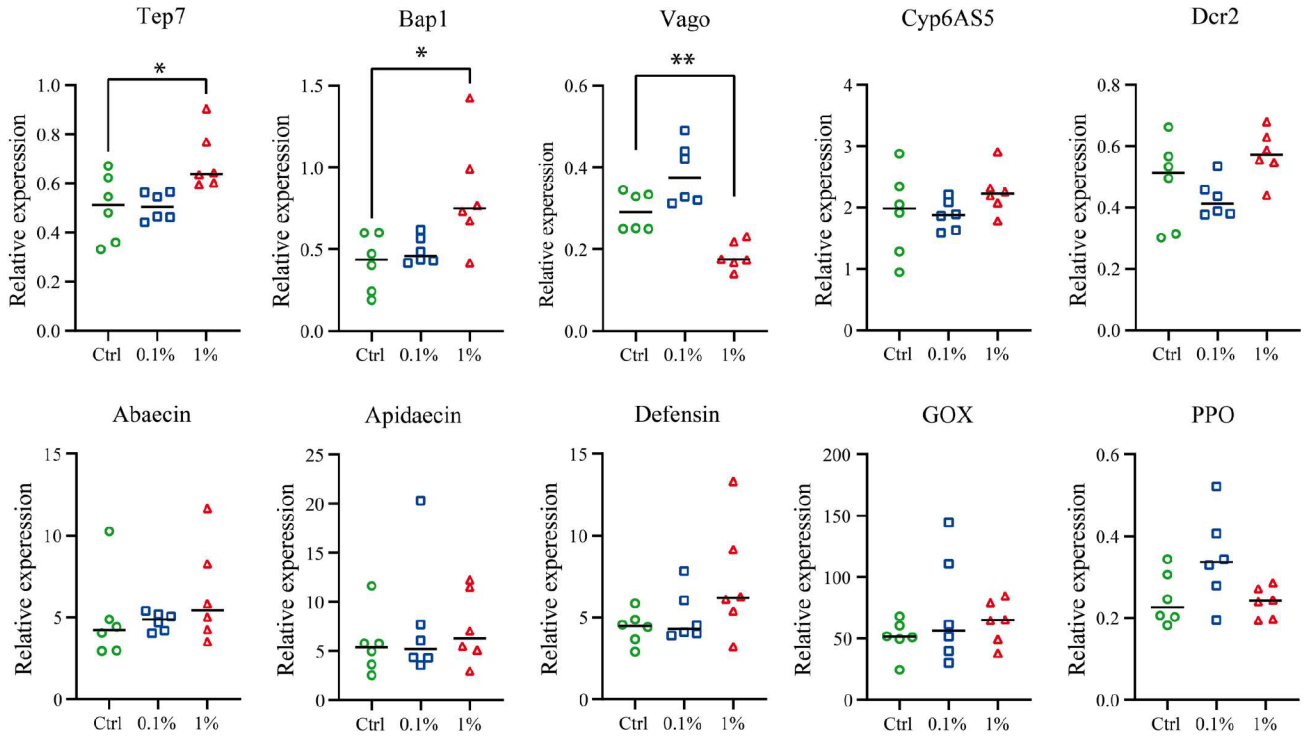

Fig. 4. *Cortinarius caperatus* significantly changed the expression of several immune genes in cage experiments 14 days after application. Feeding honey bees in cages with 1% mushroom extract in sugar syrup significantly increased the expression of Tep7 ($p = 0.0300$), Bap1 ($p = 0.0241$) and decreased the expression of the Vago ($p = 0.0010$). The graphs display relative gene expression values for individual honey bee samples and mean with ± SD of each tested group. Statistical analysis was performed using unpaired *t*-test.

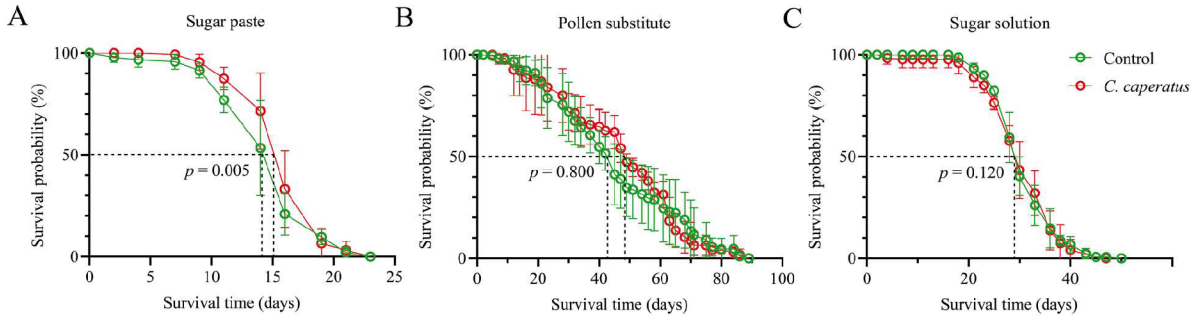

Fig. 5. *Cortinarius caperatus* did not shorten the lifespan of honey bees. (A) Feeding of 1% mushroom extract in sugar paste, (B) in dry food supplement, and (C) in sugar syrup did not shorten the lifespan of caged honey bees. The dotted line represents time when half of all honey bees died. Circles represent the mean value of living honey bees within each tested group, error bars indicate ± SD. Colors distinguish the tested groups (control group in green and experimental group in red). Groups fed with sugar paste and food supplement included 3 cages per condition, the group fed with sugar syrup included 4 cages per condition. Each cage contained 50 honey bees. Statistical analysis was performed using Cox regression.

3.3. The effects of *C. caperatus* alcohol extract on the DWV viral load and expression of selected immune genes in field experiments

For *in vivo* validation of the antiviral effect of *C. caperatus* extract, a sugar solution with 1% mushroom extract was directly applied to honey bee colonies as part of standard late-summer feeding in August. Thirty days after the start of feeding, hives with mushroom treatment showed significantly lower DWV levels than control hives ($p = 0.0418$) (Fig. 6A). Viral titers detected in mushroom treated colonies 30 days after the start of feeding were similar to the DWV levels before the application of feeding. On the other hand, the average DWV titer in control hives increased as expected. These results correlate with our results obtained with caged bees, where the development of DWV infection was blocked after the administration of *C. caperatus* in sugar solution (Fig. 2A). An even distribution of *Varroa* mite infestation among hives in the experimental and control groups was confirmed (Fig. 6B). Finally, 30 days after the application of *C. caperatus* to the colonies, we also analyzed the expression of the same set of selected immune genes as in the cage experiments but we could not detect significant differences between the experimental group and the control group of hives at this time point (Fig. 7).

3.4. Searching for residues of *C. caperatus* in honey harvested from alcohol extract treated colonies

Although *C. caperatus* is a mushroom suitable for human

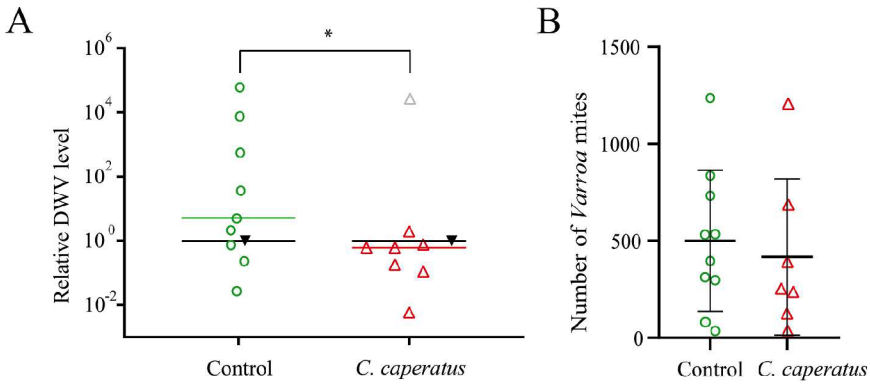

Fig. 6. *Cortinarius caperatus* reduced levels of DWV in field experiments.The level of DWV was significantly lower in colonies fed with sugar syrup supplemented with 1% mushroom extract compared to control colonies ($p = 0.0418$). Relative DWV levels are plotted as ratios of normalized values for individual honey bee samples to normalized values at time 0 (black triangles). Green and red horizontal lines represent medians of the honey bee groups, whereas black horizontal lines depict the normalizing value 1. Groups of samples are distinguished by colors and shapes of corresponding marks (black triangle for time 0, green circle for control group and red triangle for group treated with 1% mushroom). The control group included 10 colonies and the mushroom treatment group included 8 colonies. The mushroom-treated group contained one outlier sample (grey color) that was removed from statistical analysis. (B) The level of *Varroa* mite infestation did not differ between the experimental and control groups. The graph shows the number of *Varroa* mites detected in individual colonies and the mean with ± SD. Statistical analysis was performed using Mann-Whitney *U* test.

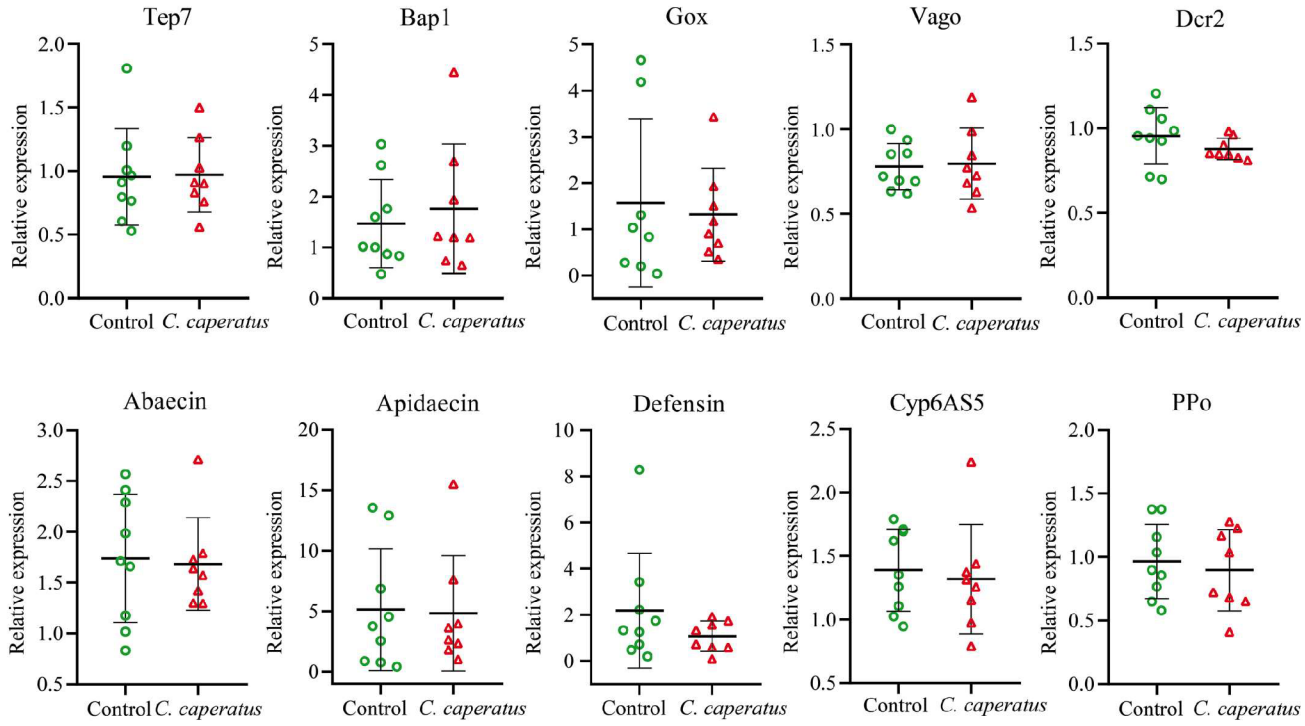

Fig. 7. *Cortinarius caperatus* did not change the expression of immune genes in honey bee colonies 30 days after application. The expression level of selected immunity related genes did not differ 30 days after the start of feeding colonies with 1% mushroom extract in sugar syrup in field experiments. The graphs display values of relative genes expressions for individual honey bee samples and mean with ± SD of experimental and control groups. Statistical analysis was performed using unpaired T-test.

consumption it would not be desirable to find any residues of the mushroom in the bee products harvested from *C. caperatus* treated colonies. To test for such residues we added the *C. caperatus* alcohol extract to the winter supplementary feeding in August and we took samples of honey from the colonies during the usual honey harvest in June the following year (Fig. 1). As revealed by GC–MS chromatogram of TMS-derived honey samples the control and mushroom treated colonies had a nearly identical GC profile and we could not detect any residues of *C. caperatus* in the mushroom treated colonies (Fig. 8).

4. Discussion

The main aim of this study was to assess the efficacy of *C. caperatus* as a treatment against the most common honey bee virus, the DWV. The results of our study indicate that the progression of DWV infection could be prevented by administering sugar syrup containing an alcohol extract of this mushroom or by application of the mushroom powder, with no negative effects on the lifespan of honey bees. This finding is important because it highlights the potential of *C. caperatus* as an economical and

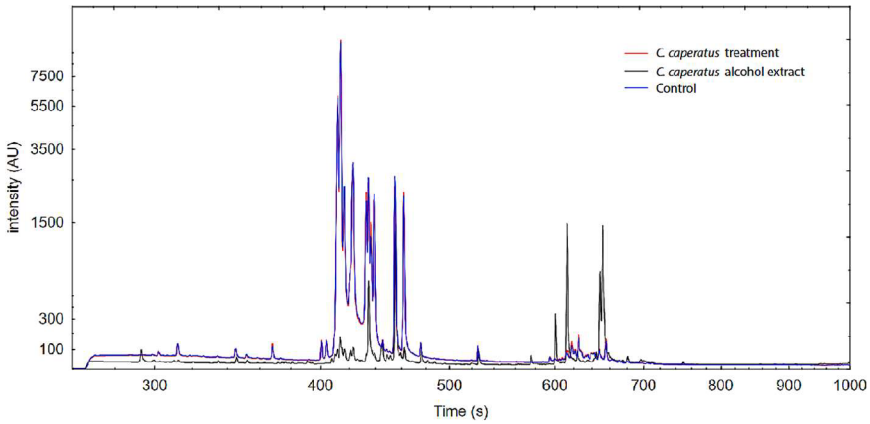

Fig. 8. No residues of *Cortinarius caperatus* were found in honey of mushroom treated colonies. *C. caperatus* alcohol extract was added to colonies during the winter supplementary feeding in August and residues in honey were tested in samples from June the following season. GC–MS chromatogram of TMS-derivatives of honey from control colonies (blue line), honey from *Rosites caperatus* treated colonies (red line) and pure *C. caperatus* alcohol extract (black line). Equal amount of each sample was introduced in gas chromatograph, Note that both axis are semi-logarithmic.

readily available antiviral treatment for honey bees. Therefore, the results of this study could have significant implications for the beekeeping industry and the health of honey bee colonies in general.

We observed that the extract as well as the dry powder of *C. caperatus* inhibits the development of DWV infection and it is safe for honey bees. Similar observation was reported in a study by Stamets et al. (2018), where an alcohol extract of *Ganoderma lucidum* and *Fomes fomentarius* reduced the levels of DWV and LSV viruses. Our study thus extends the repertoire of mushrooms carrying the potential to be an effective and low-cost antiviral treatment in beekeeping. In addition to mushrooms, phytochemicals commonly present in nectar have also been shown to effectively reduce DWV levels (Palmer-Young et al., 2017). However, the usage of *C. caperatus* may be a safer alternative as some of these substances, i.e. thymol and clove oil have been shown to reduce honey bee lifespan and increase the probability of premature death (Palmer-Young et al., 2017). Similarly, feeding honey bees with grape pomace powder has proved effective in reducing DWV titers in cage experiments with DWV-injected honey bees (Pascual et al., 2023). The antiviral mechanism of this treatment is probably associated with the ability of the orally administrated grape pomace powder to maintain the level of Relish transcriptional activity comparable to non-infected controls. However, as opposed to our study, the effects of grape pomace powder were not tested in field conditions.

Several mushrooms have been shown to contain compounds that suppress viral infections in various biological systems (Seo & Choi, 2021). *C. caperatus* contains the protein RC-83 that effectively inhibits multiple mammalian viruses (Yan et al., 2015; Piraino & Brandt, 1999). However, the maceration of the mushroom in high percentage ethanol and the oral administration of the treatment, followed by the digestion process, most probably destroys the RC-83 protein in the context of our experiments. This suggests that *C. caperatus* may contain other antiviral compounds whose activity is preserved during alcohol maceration. For example, *C. caperatus* was reported to contain phenolic compounds (Ridwan et al., 2018; Nowacka et al., 2014) that are well-soluble in ethanol (Lohvina et al., 2022) and whose antiviral properties have been well-documented (Seo & Choi, 2021). The detection of other potential antiviral compounds from *C. caperatus* is worth further investigation.

Antiviral substances can target multiple mechanisms by which viruses enter and replicate within cells or activate the host's antiviral mechanisms (Kausar et al., 2021). Our examination of certain immune genes revealed elevated expression of Tep7 and Bap1 genes and decreased expression of the Vago gene. Thioester-containing proteins (Teps) are proteins involved in the immune system and are regulated by the JAK/STAT pathway in hemocytes (Levashina et al., 2001). They are structural and functional homologs of mammalian complement as they bind pathogenic bacteria and promote phagocytosis (Shokal & Eleftherianos, 2017) or stimulate the Toll pathway to produce antimicrobial peptides and reduce invading pathogens (Dostálová et al., 2017). Teps also exhibit antiviral activity against the Dengue virus and West Nile Virus in the mosquito *Aedes aegypti* (Weng et al., 2021; Cheng et al., 2011). These proteins are not well-studied in honey bees; however, Brutscher et al. (2017) reported increased expression of Tep7 after the infection of honey bees with SINV virus. Moreover, the upregulation of Tep7 has been associated with the upregulation of Bap1 (McMenamin et al., 2021), another antiviral protein with unknown mechanisms of actions. Our observation of increased expression of Tep7 and Bap1 in mushroom-treated honey bees suggests that compounds from *C. caperatus* activates the JAK/STAT pathway leading to the upregulation of Tep7 and Bap1, either as co-regulation or as a part of a positive feedback loop. With the limited information we currently have, we can only speculate if the inhibition of the development of DWV infection was promoted directly by the virucidal action of Tep7 and Bap1 or indirectly by modulating other immune pathways to produce antiviral effectors. Therefore, further investigation is required to elucidate the mechanism of action of the alcohol extract of *C. caperatus*.

Although the treatment with *C. caperatus* extract significantly inhibited the development of DWV infection in honey bee colonies *in vivo*, we only detected changes in immune gene expression in the cage experiments but not in the field experiments. We can speculate that the expression changes may have appeared sooner than 30 days after the application of the mushroom treatment to the colonies when we sampled bees to analyzed gene expression. Therefore we were able to detect changes in expression when an earlier time point of 14 days was analyzed in our cage experiments. Moreover, the cage experiments are better experimentally controlled, with bees of the same age and same feeding regime. Additionally, the honey bees in the field experiment faced other external immune challenges, such as *Varroa* mite parasitism that the caged bees in the laboratory conditions did not encounter.

Importantly, colonies that were supplemented with the mushroom extract in their winter feed in August did not show any residues of *C. caperatus* in their honey samples obtained during the usually honey harvest in June the following year. This is not surprising as colonies consume their winter stores by the spring. In fact, as the control and mushroom treated colonies were located at the same apiary the GC–MS profile of their honey samples were nearly identical. These results suggest that administration of *C. caperatus* into the winter feed should not affect the quality of the honey harvested in the next season. However, the risk of potential residues of the mushroom in the bee products

harvested for human consumption should always be considered by beekeepers before applying any homemade antiviral treatment based on *C. caperatus*. Moreover, while this study shows no negative effect on lifespan of bees that consumed *C. caperatus* extract, negative effects on the individual or colony level can not be fully excluded. The findings of this study have significant practical implications. As *C. caperatus* is easily identifiable and common in temperate forests, the alcohol extract of this mushroom represents an effective, easily available and low-cost antiviral treatment for honey bee colonies. Our findings also show that honey bees fed with mushroom extract in sugar syrup only had approximately half the life expectancy compared to honey bees fed with mushroom extract mixed with a pollen substitute that has a high protein content. This highlights the importance of providing proper nutrition to honey bees along with antiviral treatments, either by providing natural supply of pollen or by feeding protein-rich pollen substitutes (Noordyke & Ellis, 2021; Hoover et al., 2022). It has been shown that nutritional stress can negatively impact immune responses and other physiological functions in honey bees (Castelli et al., 2020; DeGrandi-Hoffman & Chen, 2015; Alaux et al., 2010). Therefore, we recommend combining the antiviral treatment with protein as well as sugar supplementation during the season when adequate food supplies may be scarce.

Certain practical limitations exist regarding *C. caperatus* collection in nature. The season of growth of this mushroom is restricted only to a few months at the end of summer and the beginning of autumn (Akata et al., 2015), meaning that the mushroom is not always available. Also, its growth is dependent on adequate levels of precipitation and humidity. Additionally, *C. caperatus* is a mycorrhizal species (Akata et al., 2015), making indoor cultivation and industrial production of the extract unfeasible. Consequently, the commercial use of this mushroom for antiviral treatments is challenging. Nonetheless, our results suggest that *C. caperatus* can serve as an effective homemade antiviral remedy for beekeepers seeking to treat viral infections in their hives.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinsphys.2023.104583.

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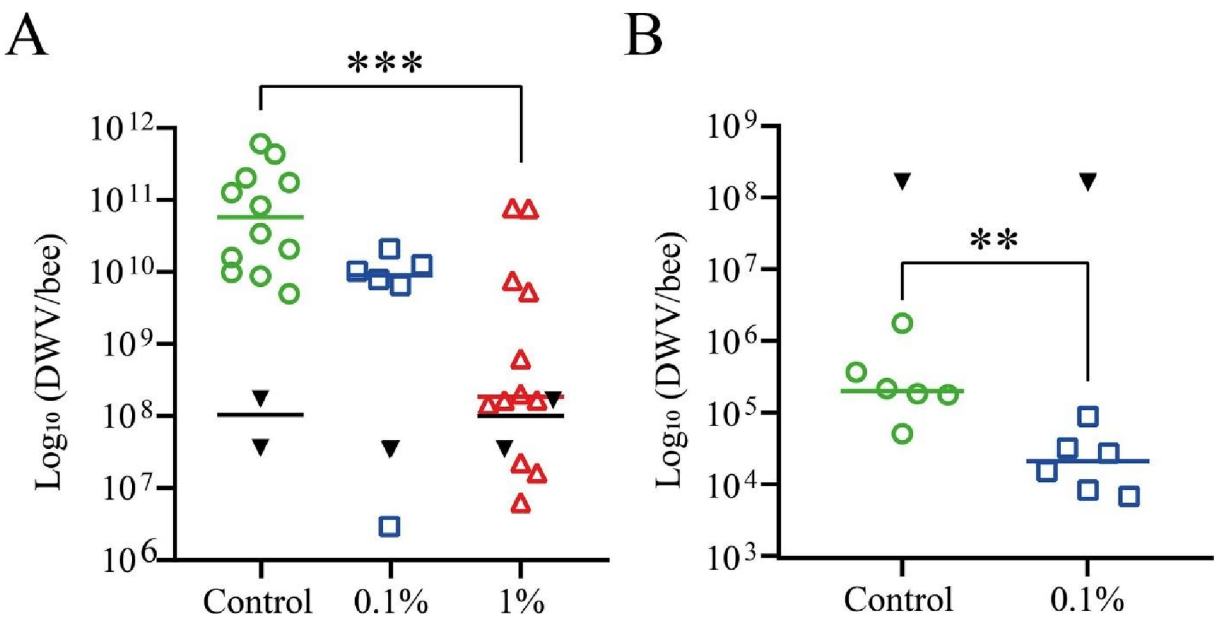
Supplemental information

Alcohol extract of the gypsy mushroom *Cortinarius caperatus* inhibits the development of Deformed wing virus infection in western honey bee (*Apis mellifera*)

Karolína Svobodová, Václav Krištůfek, Jiří Kubásek, and Alena Krejčí

Supplementary table 1. List of used primers.

Target	Source	Name	Direction	Sequence
Actin	This study	AmActin s	forward	TGCCCACACTGTCCTTTCTG
		AmActin a	reverse	AGAATTGACCCACCAATCCA
Rp49	Thaduri et al. (2019)	RP49-qF	forward	AAGTTCATTCGTCACCAGAG
		RP49-qB	reverse	CTTCCAGTTCCTTGACATTATG
Deformed wing virus	Bradford et al. (2017)	Pan-DWV_F	forward	ACGCAACCCCAGGAAT
		Pan-DWV_R	reverse	GTAGCTAATTTTACCCAATCTTTAAA
Lotmaria passim	This study	Lotpa_gGAPH_Fw	forward	TCCAGTCGATGTGCGAGGGC
		Lotpa_gGAPH_Rev	reverse	GGCCGTTCACCACGAGCACG
Nosema ceranae	This study	Noscer_rpb1_Fw	forward	TGAATCTTCATATGCCACAGAG
		Noscer_rpb1_Rev	reverse	TCTCTAAGTGTAAAAAGCCTTAGACTG
Dcr2	This study	AmDcr2 s	forward	TCCGGGACGTATGAAAGTTG
		AmDcr2 a	reverse	CAGTTGGTGATACCTCTGCTTC
Vago	This study	Vago s	forward	ATTCCAGCAGACGTTCTTCG
		Vago a	reverse	GTAGAACGGCCACAAAATGG
Tep7	This study	AmTep7 s	forward	TTTTGGAAGGACAAGAAGTGC
		AmTep7 a	reverse	ATCGGAGTATTGCGGTTCTG
Bap1	This study	AmBap1 s	forward	AATGGAATGCTCCAACTTGC
		AmBap1 a	reverse	CATGTTAATTATTTGATCTCGTATGG
Cyp6AS5	This study	AmCYP6AS5 s	forward	AATTTGCGAATCGAGGATTG
		AmCYP6AS5 a	reverse	ACGTGAATATTGGGGAGAGC
Abaecin	This study	AmAbaecin s	forward	CACTACTCGCCACGATATGC
		AmAbaecin a	reverse	GGCCATTTAATTTTCGGATTG
Apidaecin	This study	AmApidaecin s	forward	TAGTCGCGGTATTTGGGAAT
		AmApidaecin a	reverse	TTTCACGTGCTTCATATTCTTCA
Defensin	This study	AmDefensin s	forward	TGCGCTGCTAACTGTCTCAG
		AmDefensin a	reverse	AATGGCACTTAACCGAAACG
Glucose oxidase	This study	AmGOX a	forward	AAACAGGCGGTGAGAATGTC
		AmGOX s	reverse	ATCGTTGCTCCAGATGACG
Prophenoloxidase	This study	AmPPO s	forward	TTATTTATCCCGGCACATCG
		Am PPO a	reverse	TCGCGACAATAAACTGCAAC



Supplementary Figure 1. Absolute quantification of DWV in cage experiments.

(A) Honey bees fed with 1% mushroom extract in sugar syrup have significantly lower titers of DWV compared to controls ($p = 0.0004$). (B) Feeding honeybees with 0.1% dry mushroom in honey significantly reduced DWV levels ($p = 0043$). Horizontal lines represent the medians of the honey bee groups. Groups of samples are distinguished by the colors and shapes of corresponding marks (black triangle for time 0, green circle for control group, blue square for group treated with 0.1% mushroom and red triangle for group treated with 1% mushroom). Testing of mushroom extract was performed in 2 independent biological replicates (group fed 0.1% extract was not included in the second biological replicate), while the experiment with honey feeding contained a single biological replicate. Data replotted from Figure 2. Statistical analysis was performed using Mann-Whitney U test.

CHAPTER II: Gut microbiota assembly of Gotland varroa-surviving honey bees excludes major viral pathogens

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Gut microbiota assembly of Gotland varroa-surviving honey bees excludes major viral pathogens

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ABSTRACT

The spread of the parasite *Varroa destructor* and associated viruses has resulted in massive honey bee colony losses with considerable economic and ecological impact. The gut microbiota has a major role in shaping honey bees tolerance and resistance to parasite infestation and viral infection, but the contribution of viruses to the assembly of the host microbiota in the context of varroa resistance and susceptibility remains unclear. Here, we used a network approach including viral and bacterial nodes to characterize the impact of five viruses, Apis Rhabdovirus-1 (ARV-1), Black Queen Cell virus (BQCV), Lake Sinai virus (LSV), Sacbrood virus (SBV) and Deformed wing virus (DWV) on the gut microbiota assembly of varroa-susceptible and Gotland varroa-surviving honey bees. We found that microbiota assembly was different in varroa-surviving and varroa-susceptible honey bees with the network of the latter having a whole module not present in the network of the former. Four viruses, ARV-1, BQCV, LSV, and SBV, were tightly associated with bacterial nodes of the core microbiota of varroa-susceptible honey bees, while only two viruses BQCV and LSV, appeared correlated with bacterial nodes in varroa-surviving honey bees. *In silico* removal of viral nodes caused major re-arrangement of microbial networks with changes in nodes centrality and significant reduction of the networks' robustness in varroa-susceptible, but not in varroa-surviving honey bees. Comparison of predicted functional pathways in bacterial communities using PICRUSt2 showed the superpathway for heme b biosynthesis from uroporphyrinogen-III and a pathway for arginine, proline, and ornithine interconversion as significantly increased in varroa-surviving honey bees. Notably, heme and its reduction products biliverdin and bilirubin have been reported as antiviral agents. These findings show that viral pathogens are differentially nested in the bacterial communities of varroa-surviving and varroa-susceptible honey bees. These results suggest that Gotland honey bees are associated with minimally-assembled and reduced bacterial communities that exclude viral pathogens and are resilient to viral nodes removal, which, together with the production of antiviral compounds, may explain the resiliency of Gotland honey bees to viral infections. In contrast, the intertwined virus-bacterium interactions in varroa-susceptible networks suggest that the complex assembly of microbial communities in this honey bee strain favor viral infections, which may explain viral persistence in this honey bee strain. Further understanding of protective mechanisms mediated by the microbiota could help developing novel ways to control devastating viral infections affecting honey bees worldwide.

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1. Introduction

Western honey bee (*Apis mellifera*) is the most significant pollinator of flowering plants with considerable economic (Gallai et al., 2009) and ecological importance (Hung et al., 2018). The spread of the parasite *Varroa destructor* and associated viruses, however, has resulted in massive honey bee colony losses, mainly in Europe and Nothern America (Martin, 2002). To prevent these losses, a range of chemical treatments and biotechnological applications limiting the expansion of the mite population has been implemented in beekeeping practice. However, several honey bee populations have been reported to be able to survive for a long time despite no human intervention (Locke, 2016; Moro et al., 2021).

One of the best-described varroa-surviving honey bee populations was established in Gotland after several years of natural selection of colonies without beekeeping management (Fries et al., 2006; Locke, 2016). Besides the smaller size of the colonies and more frequent swarming, the adaptive traits of this population include suppression of mite reproduction allowing honey bees to effectively control mite population growth (Locke and Fries, 2011). Interestingly, this population can cope with viral infections more effectively than local varroa-susceptible honey bees, as they show lower titers of Apis Rhabdovirus-1 (ARV-1), Black Queen Cell virus (BQCV), Lake Sinai virus (LSV) and Sacbrood virus (SBV) and can tolerate high titers of Deformed wing virus (DWV) (Thaduri et al., 2018). The mechanism by which Gotland varroa-surviving bees cope better with viral infections is still not understood. However, the study of Yun et al. (2022) revealed that SBV resistance in naturally varroa-resistant *Apis cerana* is associated with the composition and diversity of gut microbiota.

The honey bee gut microbiota consists mainly of nine bacterial taxa, namely, *Bartonella*, *Bifidobacterium*, *Bombella*, *Commensalibacter*, *Frishella*, *Gilliamela*, *Lactobacillus* Firm-4 and Firm-5, and *Snodgrasella* (Kwong and Moran, 2016). It has been shown that the presence of gut microbiota plays a key role in the tolerance of viruses as dysbiotic honey bees have a significantly shorter lifespan after DWV infection compared to honey bees with established gut microbiota (Dosch et al., 2021). However, the function of the gut microbiota in viral infections in honey bees is not well understood. Studies carried out on the mosquito *Aedes aegypti* and the fruit fly *Drosophila melanogaster* showed that insect gut microbiota can modulate viral infections in several ways, i.e., by priming the immune system (Ramirez et al., 2012; Sansone et al., 2015), or releasing factors modulating defense barriers (Wu et al., 2019; Angleró-Rodríguez et al., 2017). Therefore, we hypothesized that the specific interplay between viruses and gut microbiota can be the driver of increased tolerance to DWV and improved defense mechanisms against other viruses in the Gotland varroa-surviving population of honey bees.

Modeling and analysis of microbial networks provide an inside into the structural properties and dynamics of microbial communities (Faust and Raes, 2012; Layeghifard et al., 2017). Based on the abundance data of observed amplicon sequence variants, the algorithm can reveal trait-specific co-occurrence patterns that can be visualized as networks. The resulting networks display microbial taxa that are depicted as nodes with edges symbolizing significant associations between them (Röttjers and Faust, 2018). Using 16 S rDNA sequences, the microbial community can also be explored in terms of predicted metabolic profile by aligning the sequences against the reference database (Douglas et al., 2020; Hou et al., 2021). Information inferred from networks of co-occurrences together with predicted functional profiles provide a broad-scaled overview of the studied microbial community.

In this study, we merged all available hypervariable regions of 16 S rDNA sequences from Thaduri et al. (2021) and used a network approach to further investigate the associations between gut bacterial taxa and honey bee viruses in Gotland varroa-surviving population. Even though the original study observed only minor changes in microbial diversity related to strains of honey bees (Thaduri et al., 2021), we speculated that the interactions between gut bacteria and viruses may not be recognizable from metrics of diversities. We observed that the presence of viruses completely changed the assembly of the microbial community in varroa-susceptible honey bees, while the changes of microbial structure in varroa-surviving honey bees were marginal. Our result suggests that the Gotland varroa-surviving population evolved a robust structure of gut microbiota that possibly enhances resistance to honeybee viruses.

2. Methods

2.1. Original dataset

The dataset of 16 S rDNA and values of viral loads were obtained from a previously published study whose aim was to investigate the possible contribution of the microbiota composition to the natural varroa resistance of the Gotland honeybee population (Thaduri et al., 2021). The experimental design was based on a five-stage bi-monthly comparison during a single bee season (April, June, August, September and October 2015) of the composition of gut microbiota and loads of the five most common honey bee viruses (ARV-1, BQCV, DWV, LSV, and SBV), in pooled samples of thirty 14-day old worker honey bees per colony and per sampling occasion, from each of six colonies headed by Gotland varroa-surviving queens (referred hereafter as varroa-surviving) and six matching colonies headed by regular varroa-susceptible queens (referred hereafter as varroa-susceptible), resulting in a total of 58 DNA samples (5 occasions x 12 colonies – 2 samples lost due to the premature death of one colony). The study showed strong seasonal dependence of the gut bacterial composition in both groups, with plausible biological explanations for these temporal fluctuations, but no significant systematic difference in bacterial composition between the varroa-surviving and varroa-susceptible colonies at any stage during the season (Thaduri et al., 2021). A broadly similar pattern was observed for the five viruses screened. Each virus had unique seasonal fluctuations, but with little systematic difference between the varroa-surviving and varroa-susceptible colonies, with the exception of significantly elevated SBV levels in the susceptible colonies during the early and late part of the season, slightly elevated DWV levels in the susceptible colonies during the spring and summer and elevated LSV levels in the varroa-surviving colonies during the middle part of the season (Thaduri et al., 2021). Also, genetically the virus sequences varied more by seasonality than by varroa surviving phenotype.

The 16 S rDNA amplicon sequences for the 58 honeybee microbial DNA samples were produced using The Ion 16 S™ Metagenomics Kit (Thermo Fisher Scientific, Waltham, MA, USA), which allowed the simultaneous targeted amplification of V2, V3, V4, V6–7, V8, and V9 hypervariable regions of 16 S rRNA gene. The sequencing process included the ligation of adapters using Xpress Barcode Adapter kit and sequencing with the IonS5 System. The levels of honey bee viruses were measured via RT-qPCR and the resulting number of viral genome copies was calculated from the standard curves of each target (Thaduri et al., 2021). The number of varroa mites was obtained from soapy-water washes and only data from a single collecting point (August) was available and thus incorporated into our analysis.

3. 16 S rRNA amplicon sequence processing

The whole sequence data, which included multiple (V2, V3, V4, V6–7, V8, and V9) short-read amplicons sequences, were combined in the reconstruction of a near full-length 16 S marker gene using via Short MUltiple Reads Framework (SMURF) algorithm, which allows increasing the taxonomic resolution and reducing the effect of PCR biases, for a coherent community profiling (Fuks et al., 2018). All the sequence processing was performed on the QIIME 2 environment (Bolyen et al., 2019). The SMURF method was used as implemented QIIME 2 plugin q2-sidle (Debelius et al., 2021). For this purpose, the raw sequences were split by hypervariable regions according to the primer

set used for targeted sequencing, and by using the q2-cutadapt plugin. Only the forward sequences were used since there was a total overlap of forward and reverse sequences for each targeted amplicon region. Each sequence package (by regions) was denoised using the DADA2 pipeline (Callahan et al., 2016), via q2-dada2. For each target region, a reference database was prepared based on each primer set and 16 S rRNA SILVA database v.138 (Quast et al., 2012). The Silva database was previously formatted (short and duplicated sequences discarded, and degenerate positions removed) using RESCRIPt (Robeson et al., 2021). The exact sequence fragments (by regions) were extracted from the prefiltered database using qiime2 feature-classifier (extract-reads) plugin. Using q2-sidle, each set of ASVs and the respective reference sequences were aligned forming kmers, then the kmer-based alignment was mapped with full-length SILVA reference sequences to reconstruct the feature abundance table and assign taxonomy. These artifacts were then used to reconstruct the consensus taxonomic profile.

3.1. Inference of co-occurrence networks using bacterial and viral taxa

In this study, co-occurrence networks were constructed from species-level taxonomic profiles for both varroa-surviving and varroa-susceptible honey bee groups by pooling data across all sampling time points. This approach was chosen based on i) the acknowledgement that bacterial communities and viral loads can shift over time, and ii) considering that both groups exhibited similar seasonal variations, suggesting comparable impacts of seasonal changes on the gut microbiome across groups. These observations are consistent with prior findings from Thaduri et al. (2021) which noted seasonal variations within each group but not between them. This methodology enhances the statistical power to detect key bacterial-virus associations, irrespective of seasonal variations.

For the creation of "bacterial" networks, only bacterial taxa were used, while "normal" networks included also varroa-mite and honey bee viruses (ARV-1, BQCV, DWV, LSV, and SBV). The varroa-mite and viruses were incorporated into networks by adding the log-transformed values of viral loads (quantified in RT-qPCR) and mite counts (detected with soapy water washes) into the input taxonomic profiles of each honey bee sample. To construct the networks, the Sparse Correlations for Compositional Data (SparCC) approach (Friedman et al., 2012) implemented in the R studio environment (R studio team, 2020) was used. SparCC is an iterative approximation method that calculates the correlations between the underlying absolute abundances utilizing the log-ratio transformation of compositional data. Significant positive or negative co-occurrence correlations (SparCC, weight ≥ 0.5 or ≤ -0.5) were presented in the networks. For the visualization of the core taxa sub-networks, strongly connected taxa (SparCC ≥ 0.75 or ≤ -0.75) were considered. In the networks, nodes indicate bacterial taxa, viruses, and varroa-mite while edges reflect significant co-occurrence interactions between them. The visualization of the networks and the topological properties, namely the number of nodes and edges, network diameter (the shortest distance between two most remote nodes), modularity (the strength of the division of a network into modules), average degree (the average number of links per nodes), weighted degree (the sum of the weight of all the edges connected to a node), clustering coefficient (the degree to which nodes in a network tend to form clusters), were calculated and displayed using the software Gephi 0.9.7 (Bastian et al., 2009).

For the precise visualization of the differences between "normal" microbial networks in varroa-surviving and varroa-susceptible honey bees, networks were created using the Network Construction and Comparison for Microbiome (NetCoMi) method (Peschel et al., 2021) implemented in R studio (R studio team, 2020) with the SparCC weight ≥ 0.5 or ≤ -0.5.

3.2. Comparative network analysis

For the comparison of "normal" and "bacterial" microbial networks of varroa-surviving and varroa-susceptible honey bees, a statistical analysis using NetCoMi method (Peschel et al., 2021) implemented in R studio (R studio team, 2020) was performed. The similarity between networks was assessed using the Jaccard index, which was computed for the degree (the number of links per nodes), betweenness centrality (the number of times a certain node interconnects two different nodes), closeness centrality (the number of times a given node enables the shortest possible path between two different nodes), eigenvector centrality (the importance of node related to the importance of connected neighboring nodes) and hub taxa (highly interacting taxa). The Jaccard index measures the level of similarity of the nodes with a centrality score higher than the empiric 75% quartile and spans from 0 (totally different) to 1 (entirely similar). The two *p*-values P ($J \leq j$) and P ($J \geq j$) for each Jaccard's index represent the likelihood that the computed value of the Jaccard's index is "less than or equal" or "higher than or equal" to the Jaccard value expected at random. To test the dissimilarity of clustering in the networks, the Adjusted Rand Index (ARI) was calculated. The ARI values range from − 1–1, and negative or positive ARI values indicate that the clustering is lower or higher than random. Identical clustering has an ARI value of 1, whereas dissimilar clustering has an ARI value of 0. The *p*-value shows whether the value obtained differs significantly from zero (Peschel et al., 2021).

3.3. Network robustness test

To test the differences in the hierarchical arrangement of microbial networks of varroa-surviving and varroa-susceptible honey bees the stability or robustness of the networks were analyzed by *in silico* modeling the "loss of connectivity" as a function of a "systematic nodes removal". In this test, the loss in connectivity was calculated for each network following direct, cascading, degree, or random node removal methods. Direct removal was performed by the removal of the nodes of the highest betweenness centrality. The cascading effect involves first removing nodes with a high betweenness centrality; however, this is reevaluated each time a node is removed. The degree removal is based on the elimination of nodes with the highest number of links. The last type is node removal at random. The Network Strengths and Weaknesses Analysis (NetSwan) function (Lhomme, 2015) in Rstudio (R studio team, 2020) was used to calculate network robustness. Network robustness plots were created in GraphPad 8 Prism (GraphPad Software Inc., San Diego, CA, USA). To compare the robustness of tested networks, the loss in connectivity after the fraction of 0.2 nodes removal was calculated for each network and the difference between them was expressed as a delta.

The robustness of networks with the elimination of 5 bacterial taxa was also examined, in addition to the robustness of normal and bacterial networks. These networks are based on the taxonomic tables of normal networks, from which five bacterial taxa were excluded as they best matched the present viruses and varroa mite according to their centrality metrics.

3.4. Prediction of functional traits in the honey bee microbiome

The 16 S rDNA amplicon sequences from each data set were utilized to predict the metabolic profile of each sample. The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) (Landesman et al., 2019) implemented in QIIME 2 (Bolyen et al., 2019) was used to predict metagenomes from 16 S rDNA amplicon sequences. The ASVs were placed in a reference tree (NSTI cut-off value of 3) comprising over 20,000 complete 16 S rDNA sequences from prokaryotic genomes, which was then used for prediction of individual gene family copy numbers for each ASV. The predictions are based on KEGG orthologs (KO) (Kanehisa et al., 2000). Alpha diversity (Shannon's diversity index, Pielou's evenness, and richness metrics) was estimated using the q2-diversity plugin in the QIIME2 environment, the bar plots were created in GraphPad 8 Prism (GraphPad Software Inc., San Diego, CA, USA). Statistical analysis of beta diversity (beta diversity,

PERMANOVA) proceeded in Past4.03 software (Hammer et al., 2001). Betadisper function as a part of the Vegan package (Oksanen et al., 2022) in R studio (R team, 2020) was used for the construction of PCoA plot based on Bray–Curtis distance matrix. The dispersion of samples between groups was compared using Analysis of Variance (ANOVA).

For differential abundance comparisons, pathway data tables containing sequencing-read counts and pathways abundance were used as input to the R package ALDEx2 (Fernandes et al., 2014). The ALDEx2 technique applies a centered log-ratio (clr) transformation of feature counts in each sample. The clr values of metabolic pathways were compared between varroa-susceptible and varroa-surviving honey bees using the Welsch t-test. Only the metabolic pathways with statistically different clr values between varroa-surviving and varroa-susceptible honey bees were represented by the bar plots.

4. Results

4.1. Differences in microbiota assembly in varroa-surviving and varroa-susceptible honey bees

To better understand the relationship between varroa-surviving, viral infections, and gut microbiota, co-occurrence networks were used to visualize and compare the structure of the microbial communities in varroa-surviving and varroa-susceptible honey bees. In addition to bacterial nodes, the networks contained nodes representing honey bee viruses ARV, BQCV, DWV, LSV, and SBV and Varroa mite. Visual inspection of the taxonomic networks revealed that the microbiota of varroa-surviving honey bees lacks many nodes and edges present in varroa-susceptible honey bees (Fig. 1 A), a result supported by their numerical features (Table 1). Notably, a comparison of node positions in the two networks revealed the complete absence of a module in the network of varroa-surviving, compared to varroa-susceptible honey bees (Fig. 1 A). On the other hand, some taxa (e.g., *Lactobacillus* spp., and *Bifidobacterium* spp.) were conserved in both networks. The majority of

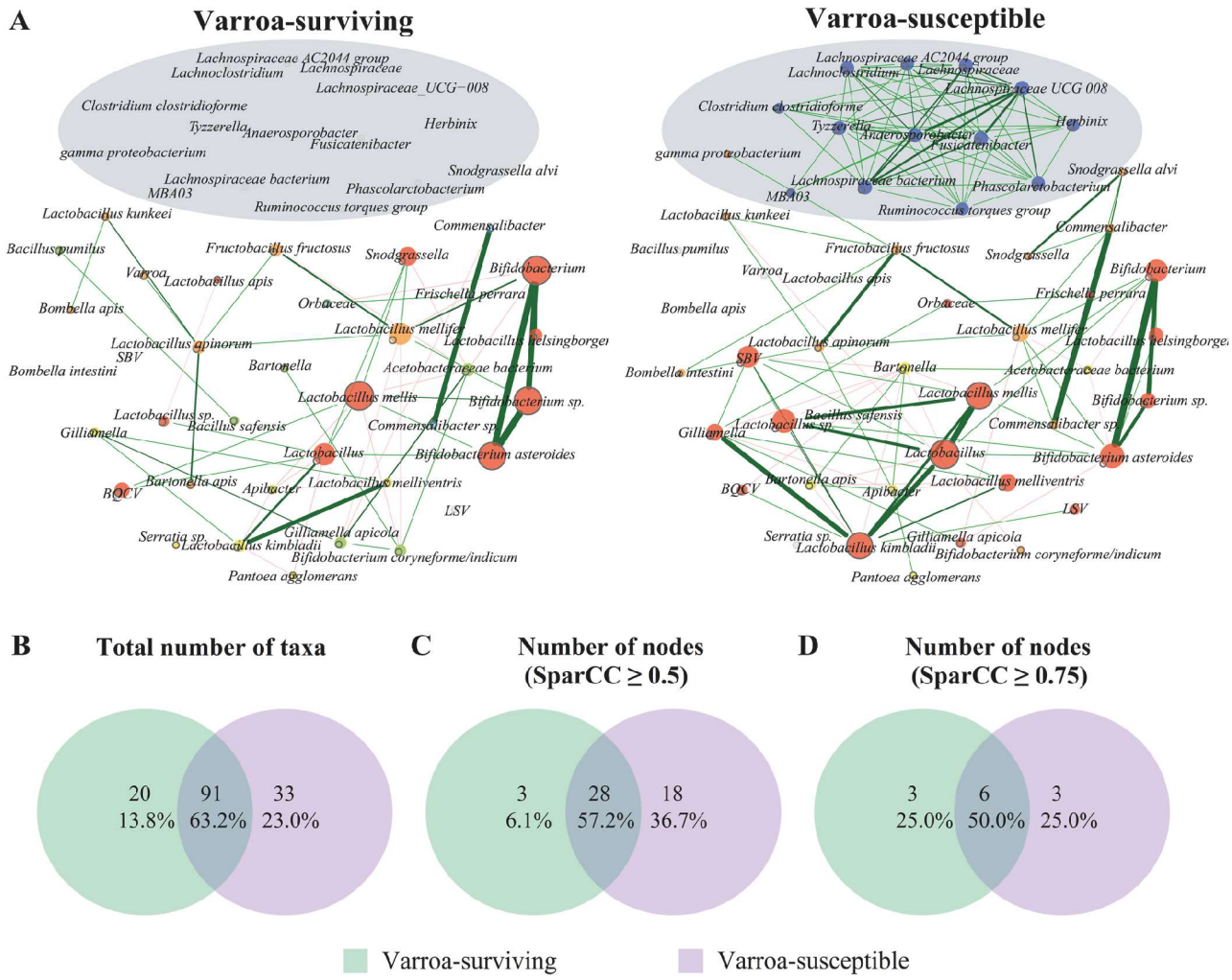

Fig. 1. Microbial community assemblies in varroa-surviving and varroa-susceptible honey bees. Co-occurrence networks (A) were extrapolated from the microbiota of varroa-surviving and varroa-susceptible honey bees. Nodes symbolize bacterial taxa and viruses with at least one connection, whilst edges represent a significant correlation between them. The width of the edges corresponds to the level of co-occurrence correlation (SparCC, weight ≥ 0.5 or ≤ -0.5). Positive (green) or negative (red) correlations are shown by the color of the edges. The colors of nodes specify clusters and modules in which taxa occur. The module unique only to one group is highlighted with a grey ellipse. The size of nodes is related to their eigenvector centrality. Venn diagrams showing the number of all taxa (B), the nodes with SparCC ≥ 0.5 (C), and the nodes with SparCC ≥ 0.75 (D) that are unique to varroa-surviving (green) or varroa-susceptible (purple) honey bees, and common to both groups (overlap).

Table 1
Topological features of varroa-surviving and varroa-susceptible networks.

Topological features	Varroa-surviving honey bees	Varroa-susceptible honey bees
Total nodes	111	124
Connected nodes	31	46
Edges	58	118
Positives	44	108
Negatives	14	10
Modularity	0.77	0.67
Network diameter	7	8
Average degree	3.74	5.13
Weighted degree	1.2	2.54
Clustering coefficient	0.47	0.63

taxa found (63.2%, total 144) were shared by both groups (Fig. 1B). However, varroa-susceptible honey bees have a higher number of unique taxa (23.0%, total 144), compared to varroa-surviving honey bees (13.8%, total 144). A similar pattern was found in the networks of weakly correlated nodes (SparCC $\geq$ 0.5), where 28 taxa (57.2%, total 49) were shared between both groups, 18 taxa (36.7%, total 49) were only found in varroa-susceptible honey bees, and 3 taxa (6.1%, total 49) were unique to the network of varroa-surviving honey bees (Fig. 1 C).

We extended the comparison to the 'core microbiota', defined here as the topological connection of the nodes with the strongest correlation between them (SparCC $\geq$ 0.75), and both groups had 3 (25.0%, total 12) unique taxa each and 6 shared taxa (50.0%, total 12) (Fig. 1D, Supplementary Table 1). Reconstruction of core microbiota sub-networks in varroa-surviving and varroa-susceptible honey bees showed shared and variable components of the core microbiota (Fig. 2). The shared motifs of the core microbiota consist of three (*Bifidobacterium* sp. (1), *Bifidobacterium* sp. (2), and *Bifidobacterium asteroids*) and two (*Commensalibacter* sp. (1) with *Commensalibacter* sp. (2)) connected nodes (Fig. 2 A). The core sub-network unique for varroa-surviving honey bees contains two motifs. The first one is based on the positive interaction of *Lactobacillus mellifer* with *Acetobacteraceae bacterium* while the second one is a negative interaction of *Lactobacillus kimbaladii* with *Lactobacillus melliventis*. In susceptible honey bees, there is only one unique motif in the variable component including four interacting partners *Lactobacillus* sp., *Lactobacillus mellis, Lactobacillus kimbaladii,* and *Gilliamella* sp. (Fig. 2B). These results suggest that the core microbial networks of two different strains of honey bees share some bacterial taxa, but also contain taxa and interactions that are unique for both of them.

Co-occurrence networks were further used to test for similarities in selected local centrality measures including degree, betweenness centrality, eigenvector centrality, and hub taxa of the networks using the Jaccard index test. The Jaccard index was higher than expected by random for closeness centrality (Jacc = 0.500, $p = 0.014$) and hub taxa (Jacc = 0.529, $p = 0.003$), while the distribution of the rest of the tested centrality measures was random (Table 2). The Adjusted Rand Index (ARI) of these two networks was 0.597 ($p < 0.001$) suggesting that the level of similarity in clusterization in these two networks was higher than expected by random. These findings, along with the topological features, suggest that the microbiota structure is similar in terms of distribution of closeness centrality, hub taxa, and clusterization, while differences found showed varroa-surviving honey bees having reduced connectivity and lower network complexity.

Co-occurrence between varroa node and other nodes in both networks was marginal and only one connection was found between

Table 2
Jaccard index for microbial networks of varroa-resistent and varroa-susceptible honey bees.

Local centrality measures	Jaccard index	P ($\leq$ Jacc)	P ($\geq$ Jacc)
Degree	0.438	0.952	0.086
Betweenness centrality	0.500	0.975	0.058
Closeness centrality	0.500	0.994	0.014 *
Eigenvector centrality	0.438	0.952	0.086
Hub taxa	0.529	1.000	0.003 * *

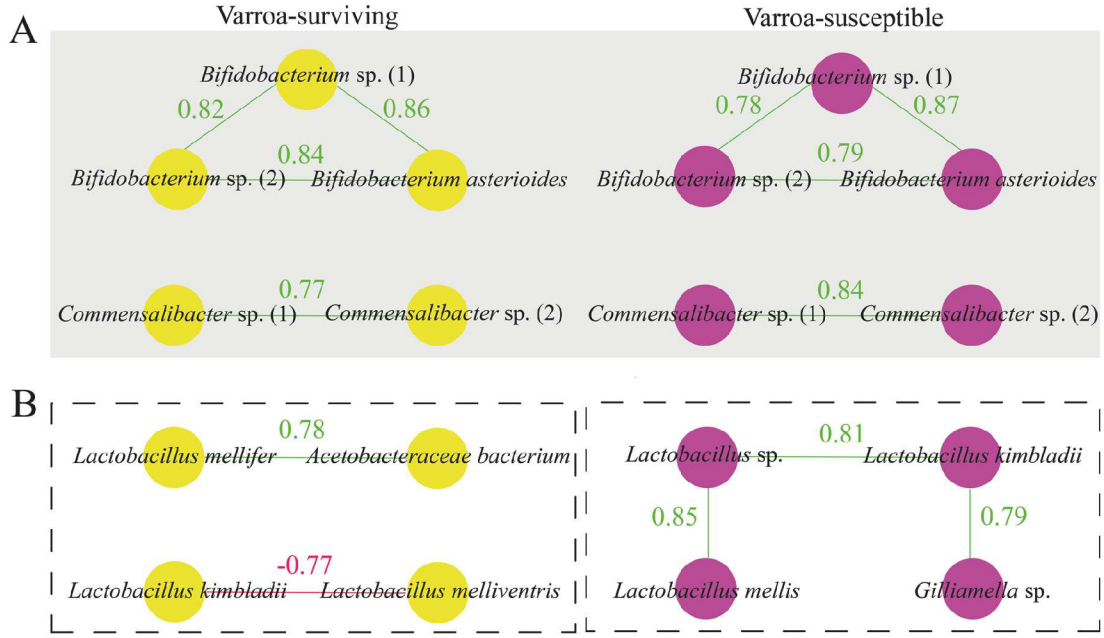

Fig. 2. Local connectivity of core taxa in varroa-surviving and varroa-susceptible honey bees. The schemes of sub-networks in the grey fields illustrate the motifs of core-taxa shared between both, varroa-surviving and varroa-susceptible groups (A). The sub-networks bordered by the dashed line represent motifs unique only for the varroa-surviving or varroa-susceptible groups (B). The color of the nodes differentiates the groups to which the nodes were identified, yellow for varroa-surviving, and violet for varroa-susceptible honey bees. The values assigned to edges express the weight of correlation between two nodes (SparCC $\geq$ 0.75 or ≤ -0.75). Positive (green) or negative (red) correlations are distinguished by the color of the edges.

Lactobacillus apinorum and varroa in varroa-surviving honey bees. Correlations between bacterial and viral nodes were observed in the networks of both varroa-surviving and susceptible honey bees (Fig. 3). In the network of varroa-surviving honey bees, bacteria-virus interactions of two viruses (BQCV and LSV) were observed (Fig. 3A), while in the varroa-susceptible honey bees, bacteria-virus correlations were found for four of the viruses (BQCV, SBV, LSV, and ARV-1) (Fig. 3B). One virus (DWV) was shown not to be involved in microbial interactions. Notably, in varroa-surviving honey bees only BQCV was found to co-occur with taxa of the core microbiota, while all bacterial nodes connected with viral nodes in varroa-susceptible honey bees were part of the core microbiota. A pattern was found for bacteria-virus interactions in which bacteria of the genera *Bifidobacterium* and *Lactobacillus* were preferentially correlated with viruses in varroa-surviving and varroa-susceptible honey bees, respectively. In varroa-susceptible honey bees, also *Gilliamella* sp. interacted with viruses. The position of viral nodes in the networks suggests they play an important role in community structure and assembly.

4.2. Role of viral taxa on microbiota assembly in varroa-surviving and varroa-susceptible honey bees

To investigate how viruses affect the bacterial community structure in varroa-surviving and varroa-susceptible honey bees, co-occurrence networks were inferred excluding the five viruses and the varroa node (referred to hereafter as 'bacterial' networks) and compared with original networks including viruses (referred hereafter as 'normal' networks). Viral nodes and varroa node removal caused topological changes in varroa-surviving (Fig. 4 A) and varroa-susceptible (Fig. 4B) networks. Notably, a module disconnected from the main network in a normal varroa-susceptible network appears connected in the bacterial varroa-susceptible network (Fig. 4B). A closer examination of network topological features confirmed the loss of 2 positive associations between taxa in varroa-surviving honey bees, and more considerable changes in varroa-susceptible honey bees, as 12 positive edges were lost, and 2 negative edges were gained in their microbial network (Table 3). This was despite viral nodes being low-ranked compared to bacterial taxa in terms of centrality measures (Supplementary Figure S1).

No changes were found, however, in the local connectivity of core taxa after the removal of viral and varroa nodes in varroa-surviving honey bees, while in the varroa-susceptible group, a new node represented by *Lactobacillus* sp. appeared in a motif unique to this group (Supplementary figure S2). This finding suggests that the removal of viral nodes affected the structure of core taxa only in varroa-susceptible honey bees, but it had not any effect on the core microbiota network in varroa-surviving honey bees. To test whether viral nodes removal influenced the node centrality distribution, Jaccard index was calculated and compared between normal and bacterial networks. Jaccard indexes were significantly higher than expected by random in all tested measures (Table 4). However, viral nodes removal caused changes in the topological distribution of betweenness and degree centrality in the networks (Fig. 5).

Jaccard index was also used for testing the similarities between centrality measures of nodes in bacterial networks of both varroa-surviving and susceptible honey bees (Table 5). Similarly, like in normal networks, the Jaccard index was higher than expected by random for closeness centrality (Jacc = 0.489, $p = 0.022$) and hub taxa (Jacc = 0.565, $p = 0.001$). Interestingly, compared to normal networks, the bacterial networks also displayed a degree-associated Jaccard index higher than expected by chance (Jacc = 0.467, $p = 0.043$). Both varroa-surviving and varroa-susceptible normal and bacterial networks showed a very similar clustering pattern, with the ARI being 0.925 ($p < 0.001$) and 0.911 ($p < 0.001$), respectively. This indicates that, in both honey bee strains, the removal of viruses from the co-occurrence network resulted in a degree distribution per node that was more similar than anticipated expected by chance, compared to normal networks.

The robustness test revealed significant connectivity loss after cascading and degree attacks in networks of varroa-susceptible compared with varroa-surviving honey bees (Fig. 6 A). The loss of connectivity after cascading and degree attacks was marginal in varroa-surviving (Fig. 6B), but strong in varroa-susceptible (Fig. 6 C) bacterial networks. Similarly, loss of connectivity associated with the removal of centrality-equivalent bacterial nodes was negligible in varroa-surviving (Fig. 6B) and considerable in varroa-susceptible (Fig. 6 C). These results suggest that viruses increase the robustness of microbial communities, notably in varroa-susceptible honey bees. However, the position of viral nodes is less important than the position of bacteria nodes.

4.3. Differences in functional profiles of varroa-surviving and varroa-susceptible honey bees

To study the differences in the metabolic functions of the gut microbiome in varroa-surviving and varroa-susceptible honey bees, we explored and compared the abundance and diversity of predicted metabolic pathways. The microbial communities of varroa-surviving and varroa-susceptible honey bees have in total 354 predicted metabolic pathways, of which 331 (93.5%) were shared by both honey bee strains, while 17 pathways (4.8%) were unique to varroa-surviving, and 6 pathways (1.7%) to varroa-susceptible honey bees (Fig. 7 A, Supplementary table S1). The diversity of microbial metabolic pathways did not reveal any difference in evenness (p=0.557), observed features (p=0.729), or Shannon index (p=0.237) (Fig. 7B). Beta diversity (Bray Curtis dissimilarity index) analysis showed no significant differences in metabolic pathways composition of varroa-surviving and varroa-susceptible honey bees (PERMANOVA, p=0.114, Fig. 7 C). Analysis of differential pathway abundance revealed two pathways with

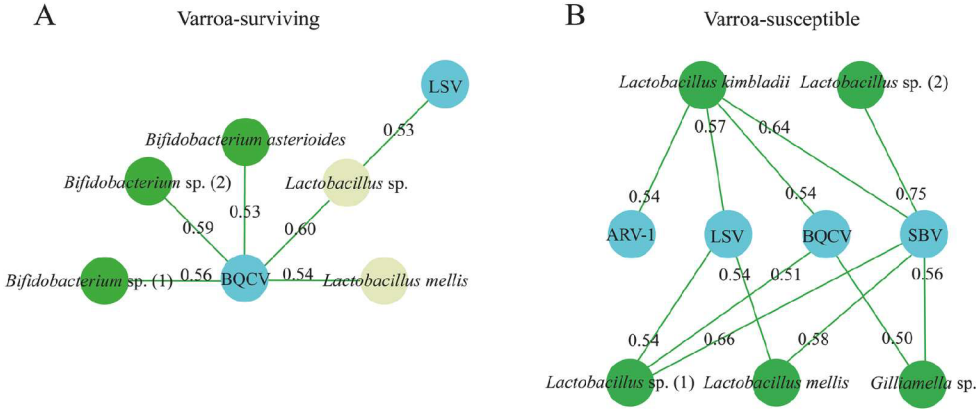

Fig. 3. Interactions between bacterial taxa and viruses. Local connections of viruses and bacterial taxa in varroa-surviving (A) and varroa-susceptible (B) honey bees. Components of local sub-networks are differentiated by the colors of nodes (blue for viruses, dark green for core taxa, and light green for non-core taxa). The strength of the correlation between two taxa is expressed by values assigned to edges (SparCC ≥ 0.5 or ≤ -0.5). Positive correlations are shown as the green color of edges. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

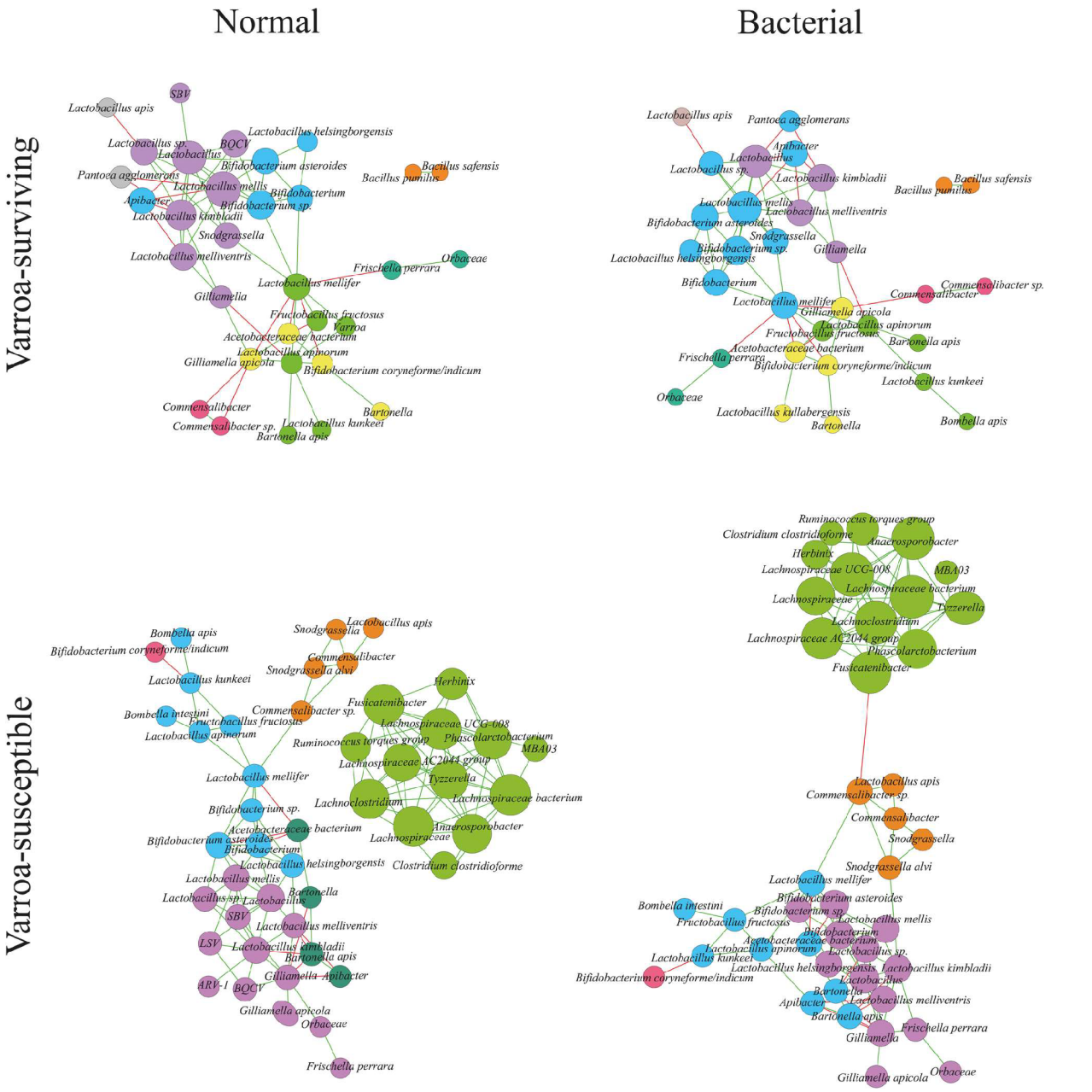

Fig. 4. The comparison of microbial arrangement in normal and bacterial networks in varroa-surviving and varroa-susceptible honey bees. The comparison of co-occurrence networks including viruses with the software-simulated co-occurrence network with the removal of viruses in varroa-surviving (A), and varroa-susceptible (B) honey bees.Nodes illustrate bacterial taxa and viruses with at least one connection, whilst connected edges represent a significant correlation between them. Positive (green) or negative (red) correlations are shown by the color of the edges. The clusters and modules in which the taxa occur are distinguished by the color of the nodes. The size of nodes is related to their eigenvector centrality.

significantly higher abundance in varroa-surviving compared with varroa-susceptible honey bees. The first was the super-pathway for heme b biosynthesis from uroporphyrinogen-III with 1.17 times higher average abundance (p=0.034), and the second one was a pathway for arginine, proline, and ornithine interconversion increased 1.06 times (p=0.046) in varroa-surviving honey bees. (Fig. 7D). None of the metabolic pathways was found to have a higher abundance in varroa-susceptible honey bees. These results suggest that metabolic profiles of varroa-surviving, and varroa-susceptible honey bees are highly similar, but with some honey bee strain-specific differences.

5. Discussion

Gotland varroa-surviving honey bee populations have been described as more resilient to viruses than local varroa-susceptible honey bees (Thaduri et al., 2018). The development and severity of viral infections are affected by the composition of the gut microbiota (Dosch et al., 2021; Mizutani et al., 2022). Therefore, resilience to viral infections in varroa-surviving honey bees is likely linked with the specific properties of gut microbiota, which may have evolved together with the varroa-resistant traits. The composition and diversity of gut microbiota of this honey bee population were recently described by

Table 3
Topological features of varroa-surviving and varroa-susceptible networks with and without viral nodes.

Topological features	Varroa-surviving		Varroa-susceptible	
	Normal	Bacteria	Normal	Bacterial
Total nodes	111	105	124	118
Connected nodes	31	31	46	41
Edges	58	56	118	106
Positive	44	42	108	94
Negative	14	14	10	12
Modularity	0.77	0.8	0.67	0.69
Network diameter	7	6	8	7
Average degree	3.74	3.61	5.13	5.17
Weighted degree	1.2	1.11	2.54	2.43
Clustering coefficient	0.47	0.46	0.63	0.6

Table 4
Jaccard index for local centrality measures in varroa-surviving and varroa-susceptible networks with and without viral nodes.

Local centrality measures	Varroa-surviving with and without viruses			Varroa-susceptible with and without viruses		
	Jaccard index	P (≤ Jacc)	P (≥ Jacc)	Jaccard index	P (≤ Jacc)	P (≥ Jacc)
Degree	0.909	1	0 * **	0.833	1	0 * **
Betweenness centrality	0.944	1	0 * **	0.810	0.999	1. E-5 * **
Closeness centrality	0.909	1	0 * **	0.800	1	0 * **
Eigenvector centrality	0.909	1	0 * **	0.846	1	0 * **
Hub taxa	0.909	1	0 * **	0.933	1	0 * **

Thaduri et al. (2021) showing fluctuations in the proportion of gut bacterial taxa during the beekeeping season. However, the distribution of bacterial taxa was generally very similar between varroa-surviving and varroa-susceptible honey bees with the exception of late spring and autumn when the distributions of *Bartonella* and *Lactobacillus* slightly differ between honey bee strains. Although diversity metrics do not show any substantial differences, the network-based analysis revealed fundamental alternations in the gut microbial interaction patterns with few important functional differences. The microbial community of varroa-surviving honey bees is noticeably simpler and less interacting with viruses, which provides structural stability without serious disruptions caused by viral infections. On the other hand, the microbial community of varroa-susceptible honey bees tends to be more affected by viruses as it includes a higher number of virus-bacteria interactions, moreover, exclusively core bacterial taxa participate in these interactions resulting in pronounced changes in the microbial community assembly. Although some insect species have been studied for the role of their gut bacteria in viral infections (Cogni et al., 2021; Donkersley et al., 2023), there is a lack of studies investigating the impact of viral infections on the gut microbiota ecological assembly and vice versa in insect. However, the global spread of the COVID-19 pandemic has prompted extensive research into microbiome-virus interactions in the field of human medicine. For example, the complex multi-omics study of Liu et al. (2022) has shown the correlation between specific microbial clusters with the severity of the COVID-19 course. Our results suggest that association between the gut microbiota assembly and viral infections can also occur in honey bees. However, while our study allowed

Table 5
Jaccard index for bacterial microbial networks of varroa-surviving and varroa-susceptible honey bees.

Local centrality measures	Jaccard index	P (≤ Jacc)	P (≥ Jacc)
Degree	0.467	0.978	0.043 *
Betweenness centrality	0.458	0.932	0.140
Closeness centrality	0.489	0.990	0.022 *
Eigenvector centrality	0.457	0.971	0.055
Hub taxa	0.565	1.000	0.001 * *

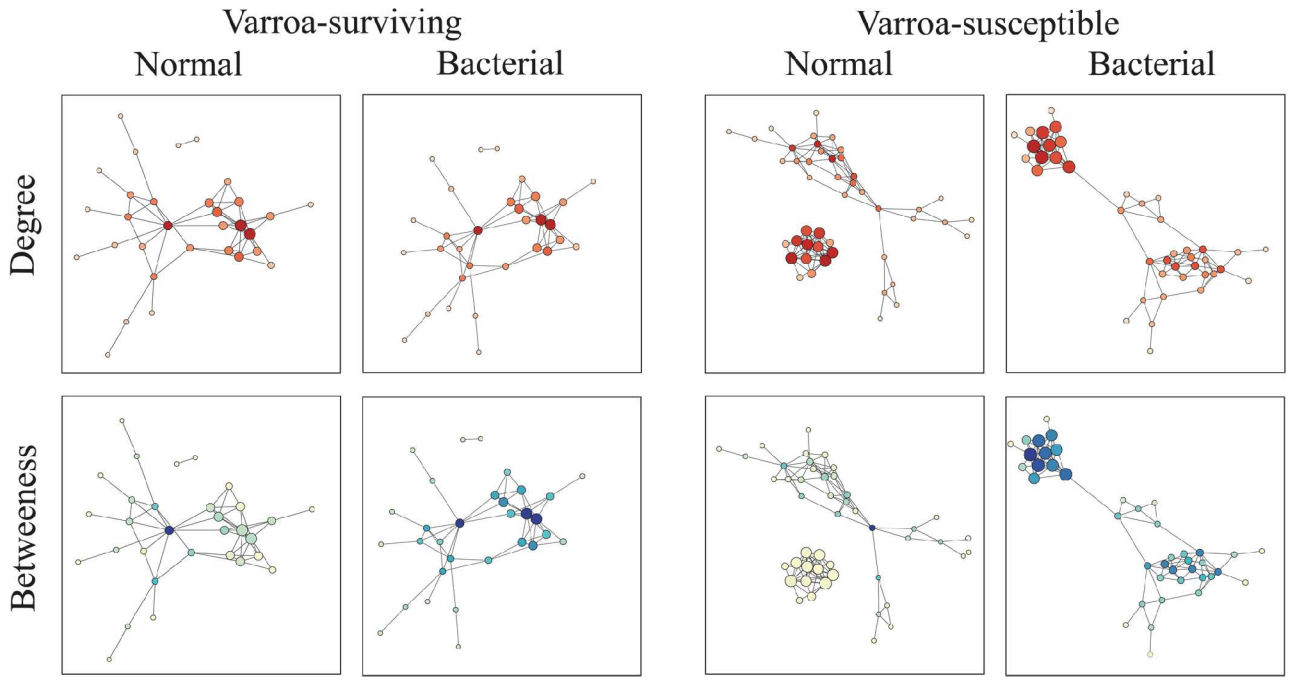

Fig. 5. The comparison of selected centrality measures of nodes in normal and virus free networks in varroa-surviving and varroa-susceptible honey bees. Nodes illustrate bacterial taxa and viruses with at least one connection, whilst connected edges represent a significant correlation between them. The color of nodes differentiates the level of centrality; the highest values are shown as red for betweenness and blue for the degree, while the lowest values are represented as light orange for betweenness and yellow for the degree. The size of nodes is related to their eigenvector centrality.

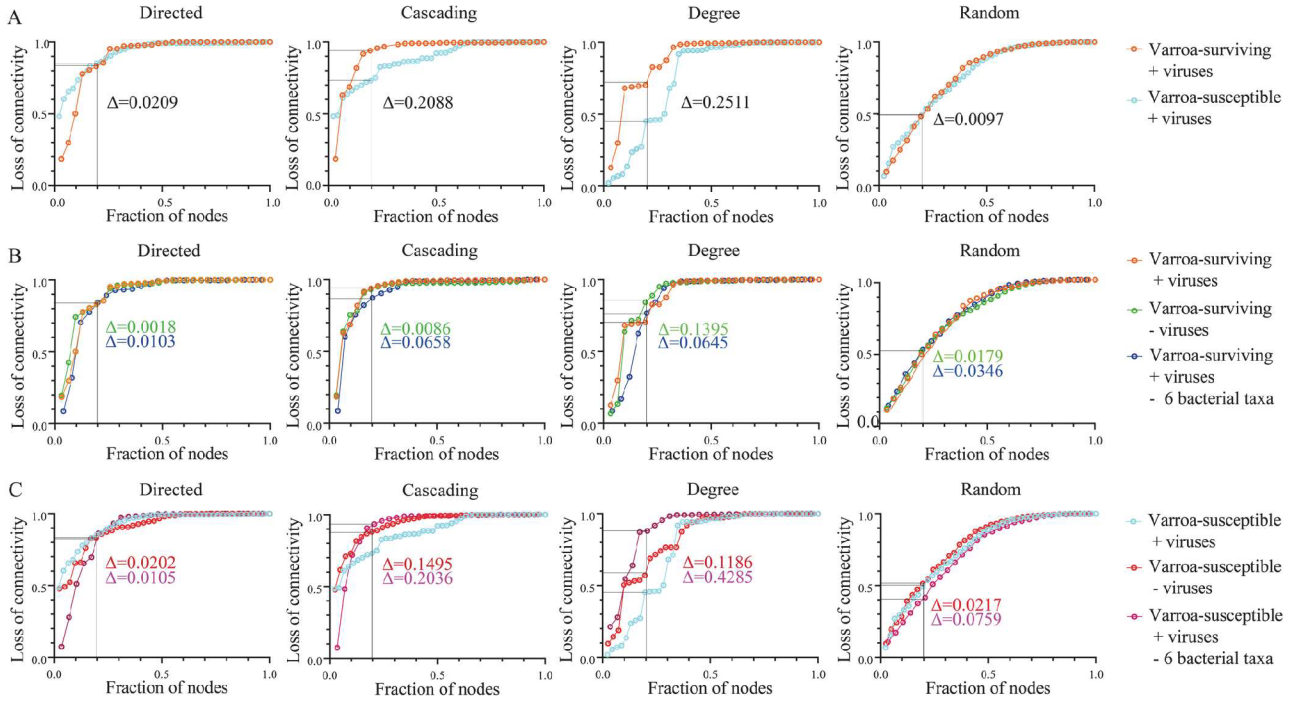

Fig. 6. Network tolerance to nodes removal. The resistance of the networks to directed, cascading, degree and random attacks was measured and compared between normal networks of varroa-surviving and varroa-susceptible honey bees (A), between normal, exclusively bacterial, and the removal of bacterial equivalents of viruses in varroa-surviving (B) and varroa-susceptible honey bees (C). Different tolerance to taxa removal is presented as delta, which was calculated as the absolute value of the difference in loss of connectivity caused by the removal of 0.20 of nodes in each network. Virus-free networks and networks with the removal of bacteria equivalents of viruses were compared with normal networks.

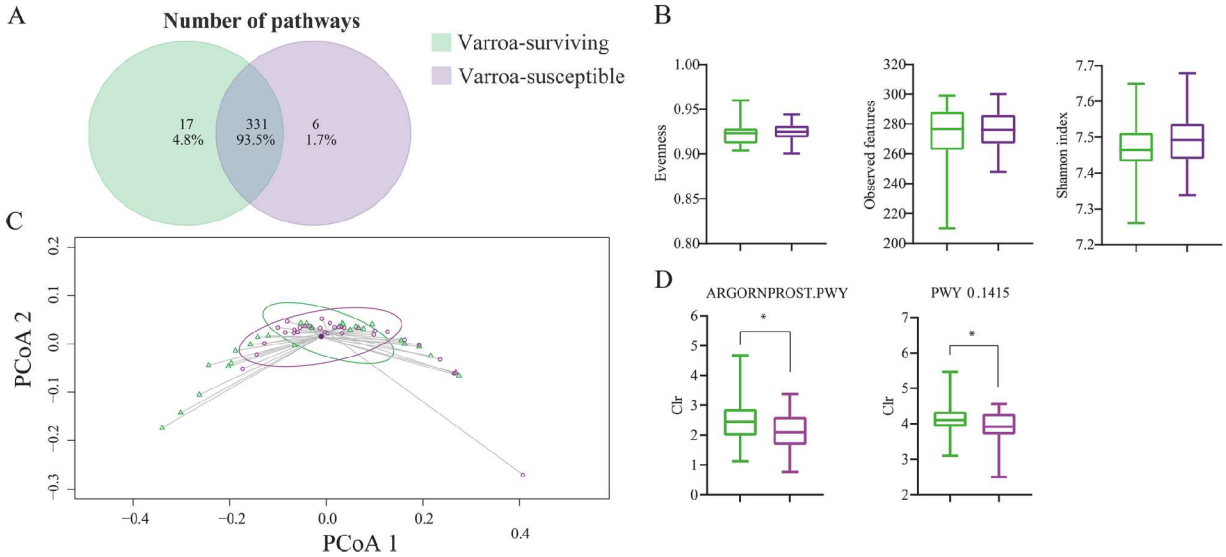

Fig. 7. Microbial metabolic pathways in varroa-surviving and varroa-susceptible honey bees. Venn diagram displaying the comparison of pathways composition between the varroa-surviving (green circle) and varroa-susceptible (purple circle) honey bees. Numerals represent the number of pathways found in each dataset and those shared by the two groups (A). Alpha-diversity of microbial pathways in varroa-surviving (green) and varroa-susceptible (purple) honey bees was observed via evenness, observed features, and Shannon index (B). Comparison of beta-diversity with Bray Curtis dissimilarity index and PERMANOVA for microbial pathways in varroa-surviving (green triangles) and varroa-susceptible (purple circles) honey bees. Small circles represent samples, and ellipses represent centroid position for each group. ANOVA test was performed and showed no difference between two groups ($p = 0.081$) (C). Boxplots of the significantly different pathways abundance in microbial communities of varroa-surviving and varroa-susceptible honey bees (D). PWY0.1415: the pathway for arginine, proline, and ornithine interconversion ($p = 0.0455$), ARGORNPROST.PWY: the super pathway for heme b biosynthesis from uroporphyrinogen-III ($p = 0.034$).

identifying associations between bacteria and viruses in the microbiota of honey bees, the causality could not be established. Specifically, it remains to be tested whether viral infection or varroa infestation modulate honey bee microbiota or whether microbiota assembly previous to infection/infestation shapes susceptibility to parasite colonization.

Although the networks of varroa-surviving and varroa-susceptible honeybees were similar in the closeness centrality and hub taxa, they also revealed important differences. The comparison showed that the microbial network of varroa-surviving honey bees consisted of a lower number of nodes and approximately half of the interactions than the microbial network of varroa-susceptible honey bees. Interestingly, despite the considerably lower number of positive interactions, the network of varroa-surviving honey bees contained a higher number of negative interactions. Coyte et al. (2021) showed that negative microbial associations are highly important for community stability because they keep each other in check and ensure that any species is not wiped out by another species. On the other hand, communities with many cooperative interactions tend to be more fragile because present species are much more dependent on each other, thus even minor disturbance can negatively affect the entire community. The robustness test revealed that the microbial network of varroa-surviving honey bees is more sensitive to cascading and degree-based node attacks compared to varroa-susceptible honey bees. This suggests that the cohesion of the microbial network of varroa-surviving honey bees relies on a few highly important bacterial nodes, thus the microbial community assembly of varroa-surviving honey bees displays a more pronounced hierarchical organization than the microbial community of varroa-susceptible honey bees.

Differences were found also at the core-microbiota level. Besides the shared motifs, the core-microbiota subnetwork of both honey bee strains contained also unique motifs. The unique motif in the core sub-network seems to be extremely connected with viral infection in varroa-susceptible honey bees, as these are the only bacterial nodes interacting with viruses (i.e., ARV-1, BQCV, LSV, and SBV). Interestingly, the viruses-bacteria interactions in the network of varroa-surviving honey bees display distinct patterns. Not only the microbiota of varroa-susceptible honey bees interacts only with BQCV and LSV, but also the interactions between BQCV occur with *Bifidobacterium* spp. of the core sub-network shared motif, and non-core *Lactobacillus* sp. The unique motif of the core sub-network is not involved in interactions with tested viruses in varroa-surviving honey bees.

Interestingly, we identified DWV as the only virus that is not involved in virus-bacteria interactions. Our hypothesis suggests that the absence of bacterial associations with DWV may be attributed to the specific mode of transmission of this virus. While viruses like ARV, BQCV, LSV, and SBV are likely acquired primarily through trophallaxis or the fecal-oral route (Yañez et al., 2020), requiring them to overcome physical and chemical barriers in the gut to penetrate cells, DWV infection primarily occurs when the varroa mite injects the virus directly into the honey bee's hemocoel (Ryabov et al., 2014). This direct injection allows for efficient entry and propagation of the virus within the honey bee's body, independent of passage through the gut. A recent study by Dosch et al. (2021) demonstrated that the gut microbiota does not impact the levels of DWV titers, but it does significantly reduce the severity of the infection. It is possible, therefore, that the gut microbiota acts as a buffer against the harmful effects of viral infection on honey bee physiology, although it does not have a direct relationship with the establishment and progression of DWV infection. Nevertheless, despite the absence of identified correlations between DWV and honey bee gut bacteria in this study, we speculate that the alternations of the immune system and metabolism caused by pervasive DWV infection may influence the composition and assembly of the gut microbiota. This effect could be masked by factors not considered in this study, such as infestations of gut parasites like *Nosema ceranae* and *Lotmaria passim*. We also observed that varroa-mite has only marginal interactions with the gut microbial network. However, it must be noted that our analysis does not include complete data about varroa mite infestation, thus this result must be considered to be indicative only.We observed that viruses play a prominent role in the structure of the microbial network of varroa-susceptible honeybees, but they are negligible from the perspective of the microbial community assembly of varroa-surviving honey bees. Our *in silico* removal of viruses showed that viruses-bacteria interactions caused a decrease in the number of negative associations and an increase in the number of nodes and positive associations in the network of varroa-susceptible honey bees. A similar trend has been observed in the gut microbial network of Malagasy mouse lemurs infected with Adenovirus (Wasimuddin et al., 2019), and in human patients with severe COVID-19 (Lai et al., 2022). The re-structurization made the network more sensitive to the removal of nodes indicating that viruses caused the equalization of nodes' centrality measures in the network. We speculate that viruses may manipulate the structure of the host microbial assembly to be robust in the organization that is the most beneficial for viruses. The study by Wasimuddin et al. (2019) also showed that the lost interactions in the Adenovirus-infected mouse lemurs' network were mainly negative interactions attributed primarily to Lachnospiraceae family. The modeled virus-free microbial community of varroa-susceptible honey bees contains a module of Lachnospiraceae bacteria having a negative association with the core taxon *Commensalibater* sp., and this association disappeared in the microbial network containing viruses. This suggests that the Lachnospiraceae module is suppressed in a virus-free state, but it acts independently on the rest of the network in case of viral infection. The Lachnospiraceae are significant producers of short fatty acids (Biddle et al., 2013), the significant nutritional substrate for gut epithelial cells (Koh et al., 2016). The Lachnospiraceae have been shown to negatively interact with pathogens in bats (Wasimuddin et al., 2018) and the decrease in Lachnospiraceae is associated with serious viral infections in humans (Sun et al., 2019; Li et al., 2022; Inoue et al., 2018). Their role in insect immunity has not been studied as far as we know. In contrast to the role of Lachnospiraceae in bats and humans, our study shows that the absence of Lachnospiraceae from the networks of varroa-surviving honey bees may be involved in tolerance to viral infections; however, revealing their role requires further investigation.

We speculate that the simplicity and the resilience to the perturbances caused by viral infections in the microbial assembly of varroa-surviving honey bees could be partly attributed to specific traits of social immunity such as propolis collecting. The study Saelao et al. (2020) has demonstrated that the high concentration of propolis within honey bee hives plays a crucial role in stabilizing the gut microbial community and limiting the abundance of Bartonella and Lactobacillus. Interestingly, the analysis of bacterial taxa abundance in Gotland varroa-surviving honey bees has shown a similar trend as the microbiota of these honey bees contained a lower abundance of Bartonella and Lactobacillus in early spring and autumn (Thaduri et al., 2021). This suggests that the presence of propolis in hives of varroa-surviving honey bees could be an important aspect of the unique features of the gut microbial assembly of varroa-surviving honeybees.

The comparison of predicted functional genes showed high overall similarity between the microbiota of varroa-surviving and varroa-susceptible honey bees with some important differences. The differential analysis revealed a significantly higher abundance of the pathway for heme b production and the pathway of arginine, proline, and ornithine interconversion in the microbiota of varroa-surviving honey bees. The heme and its reduction products biliverdin and bilirubin have been proven as antiviral agents (Singh et al., 2020). Their antiviral mechanism involves the suppression of viral replication via protease and polymerase inhibition (Zhu et al., 2010; Tsutsui and Mueller, 1987). The pathway for arginine, proline, and ornithine interconversion provides bidirectional conversion of these amino acids that are widely important for host cells (Patriarca et al., 2021; Tong and Barbul, 2004) as well as for gut bacteria (Christgen and Becker, 2019). Xia et al. (2021) showed

that mice mono-colonized with *Lactobacillus salivarius* had an enhanced interconversion between arginine and proline, which is important for the integrity of the intestinal wall as arginine regulates the intestinal tight junction (Xia et al., 2019). Therefore, the pathway for arginine, proline, and ornithine interconversion probably contributed to the thickness and integrity of gut epithelia providing a physical barrier for invading pathogens. The increased abundance of these pathways with antiviral potential may be responsible for the significantly lower titers of ARV, BQCV, LSV, and SBV in varroa-surviving Gotland honey bees previously described in Thaduri et al. (2018) or, hypothetically, also for the virus resistance observed in African and Africanized bees (Tibatá et al., 2021; Otim et al., 2020). However, in the work of Thaduri et al. (2021) the viral titers did not differ between the Gotland-derived and control colonies, suggesting that the antiviral response may not always be effective and that adapted virus tolerance may be an important component of the survival mechanisms of the Gotland honey bee populations (Locke, 2021).

In this study, we extended the findings of Thaduri et al. (2021), based on the analysis of gut microbiome diversity metrics, by employing a network approach and an analysis of microbiome functional profiles. Network analysis enables to pinpoint of the type and strengths of interactions between community members shaping the microbial ecology (Matchado et al., 2021). The networks can be expanded to include external variables like pathogenic viruses, revealing complex interacting patterns (Faust, 2021). Furthermore, microbial communities frequently adapt to external perturbations by adjusting the association networks, which may not be noticeable in alpha and beta diversities (Gao et al., 2022). Therefore, network analysis is a necessary instrument for the comparing the microbial communities.

We should note that our analysis was based on pooled samples from each colony at each time point to capture the average state of the microbial-viral network within a colony. While this approach provides an overview of broader colony trends, it may mask individual-level variations, a recognized limitation of our study design. Future work could consider individual bee sampling to better capture within-colony variability.

6. Conclusions

Our study reveals gut microbiota assembly as an important factor potentially explaining the lower susceptibility of Gotland varroa-surviving honey bees to viral pathogens, an effect that could not be detected from comparisons of bacterial diversity and composition (Thaduri et al., 2021). Differential assembly of the two honey bee strains may be linked to host genetic traits rather than the presence of the viral pathogens alone, as a viral infection was pervasive in both varroa-surviving and varroa-susceptible populations. Honey bee populations under selective pressure from viral pathogens could have evolved traits associated with host-microbiota interactions resulting in varroa-surviving honey bees being able to recruit beneficial bacteria leading to virus-tolerant assemblies. It is also possible that Gotland honey bees were colonized by novel symbionts, which may have favored differential assembly (Coyte et al., 2021), which later drove host evolution. The second explanation is also plausible, as novel symbionts can occupy important positions in the assembly of microbial communities (Wu-Chuang et al., 2022), and microbiota can influence host evolution (Brucker and Bordenstein, 2013; Kolodny and Schulenburg, 2020; Kolodny et al., 2020; Metcalf and Koskella, 2019). Regardless of the mechanism of differential assembly in Gotland honey bees (host-driven or microbiota-driven), as shown here, incoming viruses have minor importance in virus-tolerant assemblies of Gotland honey bees, in contrast to local varroa-susceptible honey bees assemblies in which viruses have a prominent position. Viral pathogens were highly nested in varroa-susceptible networks making microbiota communities more sensitive to viral nodes removal. Minimal assemblies, as those observed in Gotland honey bees, may reduce bacteria-viruses interactions by excluding bacteria that favor viral colonization via direct (e.g., positive and direct microbe-microbe interactions) or indirect (e.g., immunosuppression) mechanisms. Future research could test whether reducing bacterial assemblies in varroa-susceptible honey bees can enhance host resistance to viral infections.

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CRediT authorship contribution statement

Conception: KS, ABK and ACC; data analysis: KS, AM, DO and AWC; visualization: KS, AM and ACC; supervision: DO, ABK and ACC; software: DO; data curation: LMH; generation and provision of original datasets: ST, JM and BL; drafting of the manuscript: KS, AM and ACC; revision and edition: ST, JM, BL and ABK; reading and approval: KS, AM, DO, AWC, ST, JM, LMH, ABK and ACC.

Declaration of Competing Interest

The authors declare no competing interests.

Data availability

Raw data used in the study was uploaded to public repository, SRA: Bioproject: PRJNA941236.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2023.127418.

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Supplemental information

Gut microbiota assembly of Gotland varroa-surviving honey bees excludes major viral pathogens

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Supplementary table 1. The list of presented taxa.

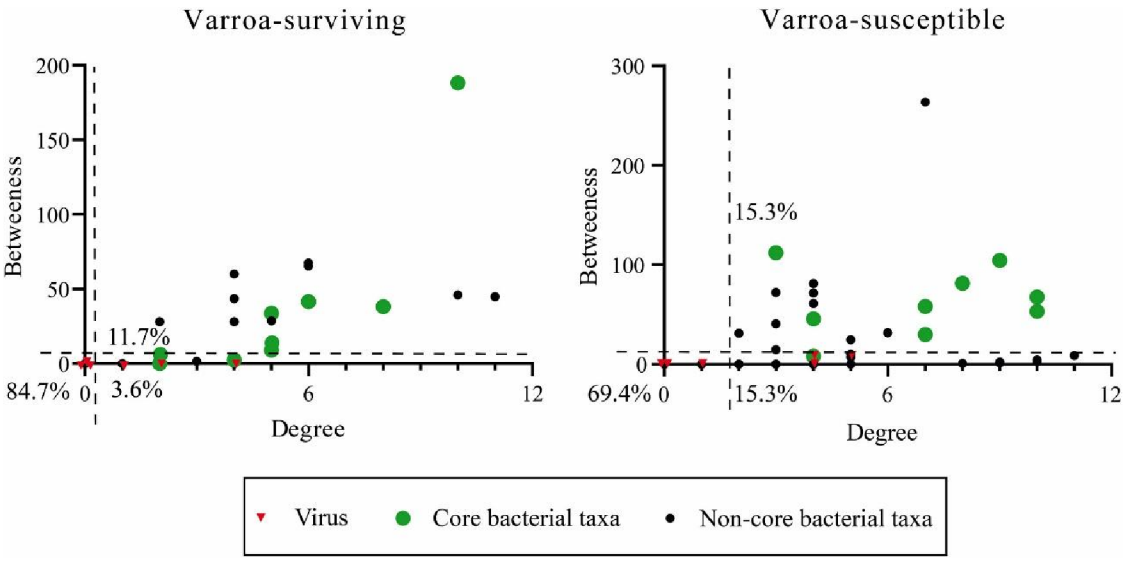
Varroa-surviving			Varroa-susceptible		
All taxa	**(SparCC ≥ 0.5 or ≤ − 0.5)**	**(SparCC ≥ 0.75 or ≤ − 0.75)**	**All taxa**	**(SparCC ≥ 0.5 or ≤ − 0.5)**	**(SparCC ≥ 0.75 or ≤ − 0.75)**
DWV	SBV	*Bifidobacterium*	DWV	LSV	*Bifidobacterium*
LSV	BQCV	*Bifidobacterium asteroides*	LSV	SBV	*Bifidobacterium asteroides*
SBV	varroa	*Bifidobacterium* sp.	SBV	ARV-1	*Bifidobacterium* sp.
ARV-1	*Bifidobacterium*	*Lactobacillus kimbladii*	ARV-1	BQCV	*Lactobacillus*
BQCV	*Bifidobacterium asteroides*	*Lactobacillus mellifer*	BQCV	*Bifidobacterium*	*Lactobacillus kimbladii*
varroa	*Bifidobacterium coryneforme| indicum*	*Lactobacillus melliventris*	varroa	*Bifidobacterium asteroides*	*Lactobacillus mellis*
Bifidobacterium	*Bifidobacterium* sp.	*Acetobacteraceae bacterium*	*Bifidobacterium*	*Bifidobacterium coryneforme| Bifidobacterium indicum*	*Commensalibacter*
Bifidobacterium asteroides	*Apibacter*	*Commensalibacter*	*Bifidobacterium asteroides*	*Bifidobacterium* sp.	*Commensalibacter* sp.
Bifidobacterium coryneforme| Bifidobacterium indicum	*Bacillus safensis*	*Commensalibacter* sp.	*Bifidobacterium coryneforme| Bifidobacterium indicum*	*Apibacter*	*Gilliamella*
Bifidobacterium sp.	*Lactobacillus*		*Bifidobacterium* sp.	*Lactobacillus*	
Rhodococcus sp.	*Lactobacillus apinorum*		*Corynebacterium*	*Lactobacillus apinorum*	
Micrococcus flavus	*Lactobacillus apis*		*Rhodococcus* sp.	*Lactobacillus apis*	
Psychromicrobium lacuslunae	*Lactobacillus helsingborgensis*		*Micrococcus flavus*	*Lactobacillus helsingborgensis*	
Apibacter	*Lactobacillus kimbladii*		*Psychromicrobium lacuslunae*	*Lactobacillus kimbladii*	
Bacillus	*Lactobacillus kunkeei*		*Dysgonomonas*	*Lactobacillus kunkeei*	
Bacillus pumilus	*Lactobacillus mellifer*		*Dysgonomonas* sp.	*Lactobacillus mellifer*	
Bacillus pumilus| Bacillus safensis	*Lactobacillus mellis*		*Apibacter*	*Lactobacillus mellis*	
Bacillus safensis	*Lactobacillus melliventris*		*Lysinibacillus* sp.	*Lactobacillus melliventris*	
Lactobacillus	*Lactobacillus* sp.		*Lactobacillus*	*Lactobacillus* sp.	
Lactobacillus apinorum	*Fructobacillus fructosus*		*Lactobacillus apinorum*	*Fructobacillus fructosus*	
Lactobacillus apis	*Acetobacteraceae bacterium*		*Lactobacillus apis*	*Lachnospiraceae*	
Lactobacillus helsingborgensis	*Commensalibacter*		*Lactobacillus helsingborgensis*	*Lachnospiraceae bacterium*	
Lactobacillus kimbladii	*Commensalibacter* sp.		*Lactobacillus kimbladii*	*[Ruminococcus] torques group*	
Lactobacillus kullabergensis	*Bartonella*		*Lactobacillus kullabergensis*	*Anaerosporobacter*	
Lactobacillus kunkeei	*Bartonella apis*		*Lactobacillus kunkeei*	*Fusicatenibacter*	
Lactobacillus mellifer	*Snodgrassella*		*Lactobacillus mellifer*	*Herbinix*	

Lactobacillus mellis	*Pantoea agglomerans*
Lactobacillus melliventris	*Frischella perrara*
Lactobacillus sp.	*Gilliamella*
Fructobacillus fructosus	*Gilliamella apicola*
Streptococcus	*Orbaceae*
Streptococcus equinus	
Paenibacillus sp.	
Lachnospiraceae	
Lachnospiraceae bacterium	
Anaerosporobacter	
Lachnospiraceae UCG-008	
Tyzzerella	
Clostridioides mangenotii	
Tissierella	
bacterium NLAE-zl-P155	
bacterium NLAE-zl-P160	
MBA03	
Phascolarctobacterium	
Acetobacteraceae	
Acetobacteraceae bacterium	
Bombella	
Bombella apis	
Bombella intestini	
Commensalibacter	
Commensalibacter sp.	
Rhizobiaceae	
Bartonella	
Bartonella apis	
Nitratireductor	
Rheinheimera aquimaris	
Snodgrassella	
Snodgrassella alvi	
Cedecea lapagei	
Cedecea davisae	
Citrobacter| *Citrobacter freundii*	
Citrobacter gillenii| *Citrobacter werkmanii*	
Citrobacter werkmanii	
Enterobacter cancerogenus	
Enterobacter kobei	
Serratia ureilytica	
Enterobacter| *Lelliottia*	

Lactobacillus mellis	*Lachnoclostridium*
Lactobacillus melliventris	*[Clostridium] clostridioforme*
Lactobacillus sp.	*Lachnospiraceae AC2044 group*
Fructobacillus fructosus	*Lachnospiraceae UCG-008*
Streptococcus	*Tyzzerella*
Streptococcus equinus	*MBA03*
Lachnospiraceae	*Phascolarctobacterium*
Lachnospiraceae bacterium	*Acetobacteraceae bacterium*
Clostridium sp.	*Bombella apis*
[Ruminococcus] torques group	*Bombella intestini*
Anaerosporobacter	*Commensalibacter*
Fusicatenibacter	*Commensalibacter* sp.
Herbinix	*Bartonella*
Lachnoclostridium	*Bartonella apis*
[Clostridium] clostridioforme	*Snodgrassella*
Lachnospiraceae AC2044 group	*Snodgrassella alvi*
Lachnospiraceae UCG-008	*Frischella perrara*
Tyzzerella	*Gilliamella apicola*
Tissierella	*Orbaceae*
bacterium NLAE-zl-P155	
bacterium NLAE-zl-P160	
MBA03	
Phascolarctobacterium	
Acetobacteraceae	
Acetobacteraceae bacterium	
Bombella	
Bombella apis	
Bombella intestini	
Commensalibacter	
Commensalibacter sp.	
Rhizobiaceae	
Bartonella	
Bartonella apis	
Nitratireductor	
Rheinheimera aquimaris	
Snodgrassella	
Snodgrassella alvi	
Cedecea lapagei	
Buttiauxella sp.	
Enterobacillus	
Enterobacter asburiae	

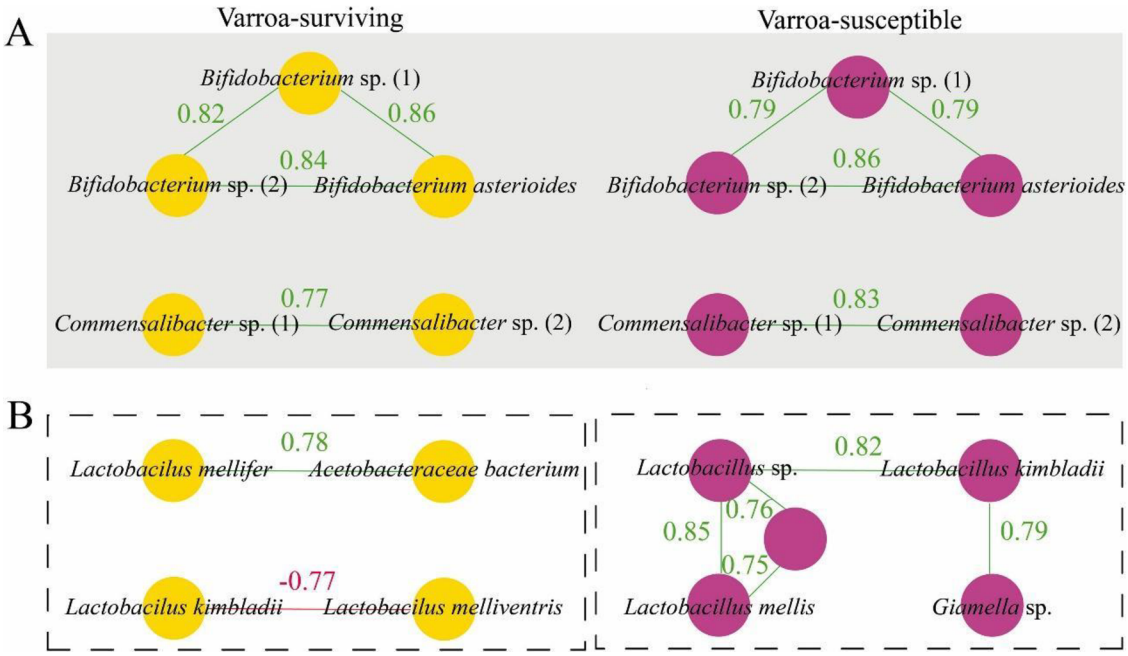
Enterobacter sp.			*Enterobacter cloacae*| *Leclercia adecarboxylata*		
Lelliottia sp.			*Enterobacter* sp.		
Citrobacter sp.			*Enterobacter* sp.| *Leclercia adecarboxylata*		
Erwinia aphidicola			*Klebsiella aerogenes*		
Erwinia toletana			*Lelliottia* sp.		
Pantoea			*Serratia ureilytica*		
Bacillus sp.| *Pantoea* sp.			*Enterobacter*| *Lelliottia*		
Pantoea agglomerans			*Klebsiella*		
Pantoea agglomerans| *Pantoea vagans*			*Klebsiella variicola*		
Pantoea ananatis			*Kluyvera*		
Pantoea brenneri			*Kluyvera intermedia*		
Phaseolibacter			*Pantoea agglomerans*		
Hafnia alvei			*Erwinia aphidicola*		
Hafnia alvei| *Hafnia* sp.			*Erwinia persicina*		
Hafnia paralvei			*Erwinia tasmaniensis*		
Arsenophonus nasoniae			*Erwinia toletana*		
Moellerella wisconsensis			*Incertae Sedis*		
Proteus penneri			*Pantoea*		
Providencia rettgeri			*Bacillus* sp.| *Pantoea* sp.		
Rahnella aquatilis| *Rahnella sp.*			*Pantoea agglomerans*| *Pantoea vagans*		
Serratia			*Pantoea brenneri*		
Ewingella americana			*Phaseolibacter*		
Hafnia sp.			*Tatumella*		
Rahnella aquatilis			*Tatumella terrea*		
Rahnella sp.			*Hafnia alvei*		
Rahnella variigena			*Hafnia alvei*| *Hafnia* sp.		
Serratia marcescens			*Hafnia paralvei*		
Serratia plymuthica			*Arsenophonus nasoniae*		
Serratia plymuthica| *Serratia sp.*			*Moellerella wisconsensis*		
Serratia sp.			*Providencia rettgeri*		
Candidatus Schmidhempelia			*Klebsiella pneumoniae*		
Frischella			*Rahnella aquatilis*| *Rahnella sp.*		
Frischella perrara			*Rahnella1*| *Serratia*		
Gilliamella			*Serratia*		
gamma proteobacterium			*Ewingella americana*		
Gilliamella apicola			*Hafnia* sp.		
Gilliamella bombi			*Rahnella aquatilis*		
Gilliamella mensalis			*Rahnella* sp.		

Orbaceae			*Serratia fonticola*		
Acinetobacter boissieri			*Serratia fonticola*| *Serratia* sp.		
Pseudomonas			*Serratia plymuthica*		
Vibrio			*Serratia* sp.		
Vibrio fluvialis			*Candidatus Schmidhempelia*		
Vibrio sp.			*Frischella*		
			Frischella perrara		
			Gilliamella		
			gamma proteobacterium		
			Gilliamella apicola		
			Orbaceae		
			Acinetobacter boissieri		
			Pseudomonas		
			Gammaproteobacteria bacterium		
			Heliconius melpomene		
			Heliconius numata		
			Vibrio		
			Vibrio fluvialis		
			Vibrio sp.		



Supplementary figure 1. Betweenness and degree centrality measures of taxa in networks.

Scatterplot showing the betweenness and degree of each non-core bacteria taxon (black dots), core bacterial taxon (green dots) and viruses and varroa (red triangles) in varroa-surviving (A) and varroa-susceptible (B) honey bees. The vertical dotted line represents the mean of betweenness of all nodes, while the horizontal dotted line represents the mean of the degree of all nodes.



Supplementary figure 2. Local connectivity of core taxa in varroa-surviving and varroa-susceptible honey bees without viruses.

Local connections of viruses and bacterial taxa in varroa-surviving (A) and varroa-susceptible (B) honey bees. Components of local sub-networks are differentiated by the colors of nodes (blue for viruses, dark green for core taxa, and light green for non-core taxa). The strength of the correlation between two taxa is expressed by values assigned to edges (SparCC > 0.5 or < −0.5). Positive correlations are shown as the green color of edges.

Chapter III: Honeybees control the gas permeability of brood and honey cappings

Jiří Kubásek, **Karolína Svobodová**, František Půta, Alena Krejčí

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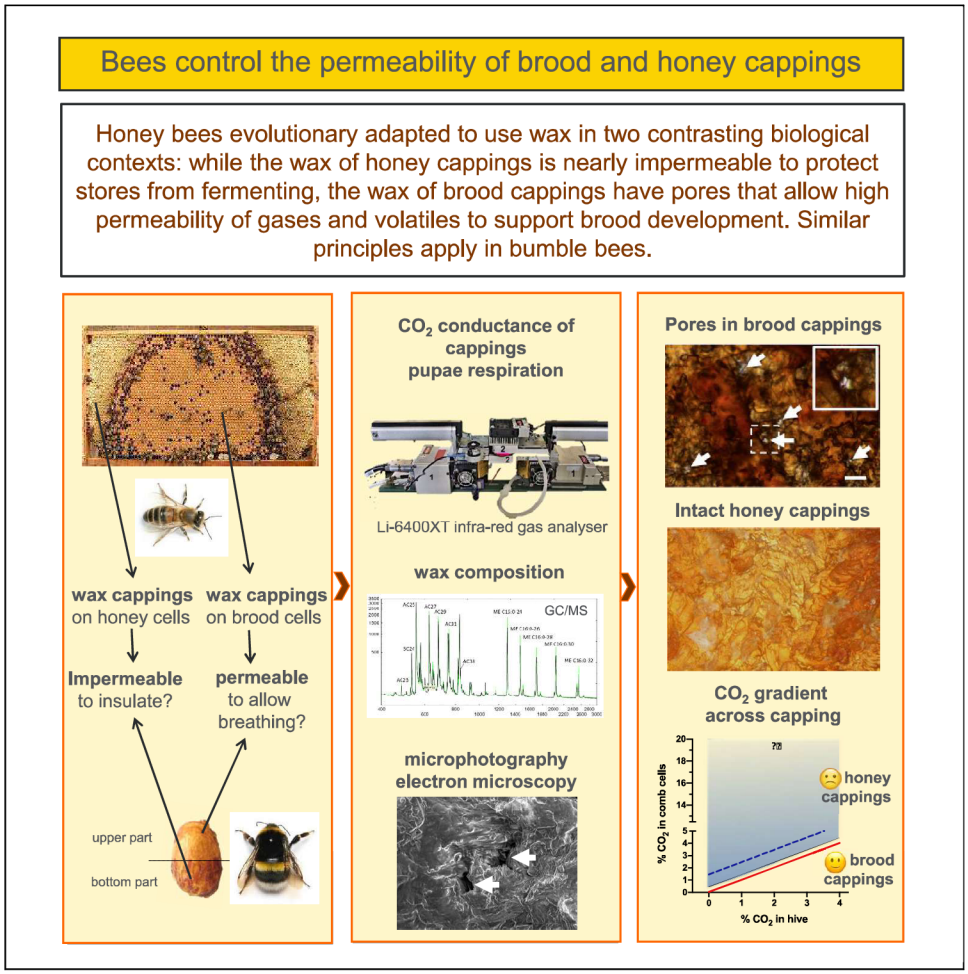
Honeybees control the gas permeability of brood and honey cappings



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Highlights

Brood cappings are well permeable to CO_2, similarly to living cells and organs

Brood cappings, but not honey cappings, contain pores

Honey cappings are poorly permeable to CO_2, as apical membrane of gut cells

Permeability of cappings as a selective pressure in the evolution of wax-building bees

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Honeybees control the gas permeability of brood and honey cappings

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SUMMARY

Some bee species use wax to build their nests. They store honey and raise their brood in cells made entirely from wax. How can the bee brood breathe and develop properly when sealed in wax cells? We compared the chemical composition and structural properties of the honey cappings and worker brood cappings of the honeybee *Apis mellifera carnica*, measured the worker brood respiration, and calculated the CO_2 gradients across the two types of cappings. We identified microscopic pores present in the brood cappings that allow efficient gas exchange of the developing brood. In contrary, honey cappings are nearly gas impermeable to protect honey from fermenting. Similar principles apply in bumble bees. Our data suggest the control of gas exchange of cappings as a selective pressure in the evolution of wax-building bees that drives their adaptation for using wax in two highly contrasting biological contexts.

INTRODUCTION

Honeybees (*Apis*), stingless bees (*Meliponini*), and bumble bees (*Bombus*) are the only known insects that have evolved the ability to use wax for building their nests. The wax cells in the nest, often associated in combs, serve for storing nectar, honey and pollen resources as well as for raising brood, besides their function as a humidity and temperature buffer[1,2] as a communication device via vibrational and smell cues[3,4] and as place for the bees to gather.[5] The use of wax cells closed with cell cappings allowed these species to store food for long periods of time, providing an evolutionary advantage to them over other bee species.

Individual wax cells can be used for storage or for rearing brood, depending on the actual needs of the colony. Once an egg hatches within a cell, the honeybee larva is incubated and fed a specific diet by the nurse bees for a species defined period of time.[6,7] The brood cell is then capped by the workers,[8] permitting the larva to spin its cocoon, transform into a pupa and complete its development inside the comb cell.[9] Bumble bees can rear several larvae together in one wax cell at the beginning of their development. The larvae of stingless bees are not fed by the adults because the cell is capped straight after egg deposition and the hatched larva utilizes pollen and honey stores deposited within the cell. Similarly, nectar is also stored in the wax cells where it is enriched and concentrated by the bees. Moreover, honeybees and stingless bees are able to store ripe honey in comb cells for several months, but only after coating them with wax cappings.[10]

The wax used to construct comb cells and their cappings is composed of more than 300 constituents, including fatty acid esters, hydrocarbons and free fatty acids.[11,12] Its lipophilic nature is well suited for storing of aqueous solutions such as honey. Similar lipidic mixtures present in insect or plant cuticles are also very impermeable to gases and water vapor.[13] Thus, wax cappings formed on ripe honey cell stores offer good protection against water reabsorption that could lead to honey fermentation because of the ubiquitously present yeast from the environment.[14] This can be considered as an important evolutionary adaptation that allows the bees to overcome long dearth periods when pollen and nectar sources are limited or absent.

Covering brood with wax capping is beneficial for proper brood development in the hive conditions by helping to buffer the humidity or temperature conditions.[1,15] *In vitro*, the honeybee pupa can develop normally without capping as long as correct temperature and highly humid conditions are maintained.[16] Within the context of the comb, the porosity and rough texture of the brood cappings serve as an important cue that helps to orient the larva longitudinally in the cell after spinning its cocoon, so that its head lies

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toward the capping and allows hatching later.[17] The importance of brood capping is also illustrated by the fact that opened brood at the stage of pupae is rarely seen in honeybee colonies, except for a short time during the recapping process[18] or during the hygienic behavior when unhealthy pupae are removed from the comb.[19]

Applying the impermeable properties of honey cappings to the wax cappings of brood cells would limit the gas exchange associated with the breathing of brood. Moreover, permeability of brood cappings is essential for the detection of volatile compounds released by varroa mites that trigger the removal of parasitized brood by the worker bees (varroa sensitive hygiene, VSH).[20] Therefore, the brood cell cappings must be much more permeable than any judgment solely inferred from the chemical properties of their constituent wax. However, although this fact can be inferred as an obvious concept, there is an extreme paucity in our collective knowledge about how such permeability is achieved. For example, no data on the diffusive conductivity of brood cappings is available in the current literature, meaning gradients of CO_2, oxygen, water vapor or other volatiles across brood cell cappings cannot be modeled at present.

To elucidate the enigma of the brood capping permeability, we have compared the chemical and structural differences between the worker brood wax cappings and wax honey cappings of the honeybee *Apis mellifera* using GC/MS analysis, microphotography and scanning electron microscopy. We have also determined the diffusive conductances of both types of the cappings by a tandem of infrared gas analyzers and measured brood respiration. Collectively, these datasets allowed us to calculate the expected CO_2 gradients across the cappings. We discuss the consequences of these differential gradients and their probable significance in instigating evolutionary adaptive behavior in the wax-nest building insects to utilize the same material for two distinction and functionally different purposes.

RESULTS

CO_2 diffusive conductance of the honeybee worker brood cappings and honey cappings

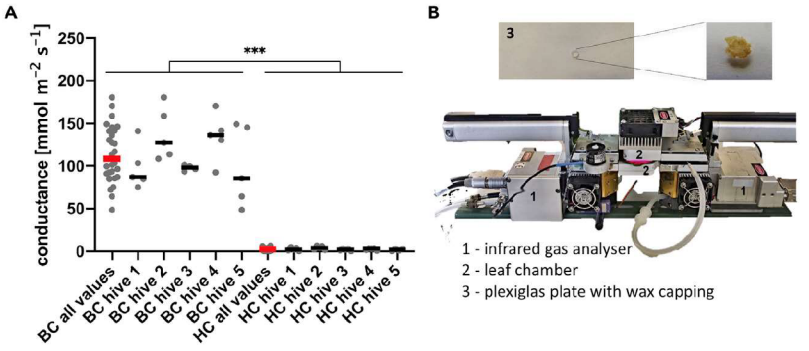
The CO_2 conductance of honey cappings was extremely low, ranging from cca 0 to 5.88 mmol m^{-2} s^{-1}, with median 3.3 mmol m^{-2} s^{-1} (n = 25). The lowest values are at the detection limit of the gas analyzer (disadvantaged further by small capping area, about 30-fold lower in comparison to default analyzer chamber area). In contrary, the brood cappings conductance ranged from 64.6 to 180.5 mmol m^{-2} s^{-1}, with median 108.7 mmolm^{-2} s^{-1} (n = 25), thus 33-fold higher than the conductance of honey cappings (Figure 1A). This difference is highly significant using Mann–Whitney U test (U = 0, Z = 6.54, $p < 0.001$).

Composition and microstructure of the honeybee cappings

We aimed to test the hypothesis that the profound differences in CO_2 conductance between the cappings could be due to the differences in their chemical composition or their structure.

Both the honey cappings and brood cappings gave very similar GC/MS profile (Figure 2A), corresponding to the typical profile of honeybee wax.[12,21,23] In agreement with the literature, we identified C27 as the most abundant n-alkane in both types of the cappings, followed by C29, C31 and C25 (Figure 2B). We also quantified palmitates and oleates monoesters that are the most abundant chemical compounds of beeswax.[12,24] According to our results, the brood cappings contained lower percentage of monoester content compensated by higher percentage of n-alkanes when compared to the honey cappings ($F_{1, 4} = 123.8$, $p < 0.001$, Figure 2B). The hives did not differ significantly when tested as a random factor ($F_{4, 23.7} = 2.20$, $p = 0.100$).

At the same time, we found substantial differences in the outer and inner surfaces of both the cappings by optical microphotography and by the scanning electron microscopy. The honey cappings have a smooth surface on their outer side whereas their inner surface is more rough (Figures 3C, 3D, 4C, and 4D). They look as a compact sheet and only very rarely contain visible pores, in agreement with their low CO_2 conductance. The brood cappings are rough on both their surfaces. As expected, their inner surface is connected with the fibers of the cocoon that the larva spins after capping. Interestingly, there are pores coming across the cappings (Figures 3A, 3B, 4A, and 4B). They are randomly scattered across the capping, often with irregular shapes, and the area of an individual pore covers 753 μm^2 as a median value (Figure 3E, n = 35 cappings). The corresponding pore diameter spans 4–53 μm, with the median value of 31 μm (Figure 3F). They are larger than the average size of bacterial or fungal cells (e.g. the microsporidian *Nosema*) but they are substantially smaller than the adult parasitic *Varroa* mite (Figure 3F). The median of the total area that is covered by pores is 0.06 mm^2 per capping, representing 0.29% of the capping surface.



C

	mol.m-2.s-1	*cm.s-1*	
brood capping	0.048 - 0.181	0.12-0.46	this study
rat cardiomyocyte plasma membrane	0.039 - 0.09	0.1 - 0.23	34
human erytrocyte plasma membrane	0.023 - 0.059	0.06 - 0.15	35
leaf surface with stomata fully open	0.14 - 0.47	0.35 - 1.22	37
honey capping	0.07 - 3.52 x 10^{-3}	0.17 - 8.9 x 10^{-3}	this study
apical membrane of guinea-pig colon	0.30 - 0.59 x 10^{-3}	0.77 - 1.5 x 10^{-3}	36
astomatous plant cuticle/leaf surface wih stomata closed	0.07 - 7.1 x 10^{-3}	0.17 - 17.3 x 10^{-3}	39

Figure 1. CO_2diffusive conductance of cappings

(A and B) Worker brood cappings 'BC' and honey cappings 'HC' were dissected from the same combs and their conductance for CO_2 measured by infra-red gas analyzers (5 hives, 5 brood cappings and 5 honey cappings per hive). Individual data points with medians. Mann–Whitney U test ($Z = 6.54$, $p < 0.001$). *** indicates $p < 0.001$ (B) Experimental setup of the tandem of Li-6400XT infra-red gas analyzers. Plexiglass plate with a wax capping is inserted in between the two leaf chambers.

(C) Comparison of CO_2 diffusive conductances of cappings with conductances of various biological systems. See also Figure S1.

Respiration rate of developing honeybee pupae

To be able to determine the CO_2 concentrations in the capped brood cell we first needed to accurately assess the respiration rates of the developing pupae. As it is apparent from Figure 5A, the 10-day-old pupae (4 days after capping) produced 1.98 to 2.69 µmol CO_2 h^{-1} per pupa (median 2.39, n = 25) that represents respiration rate 13.99 to 18.75 µmol g^{-1}FW h^{-1} (median 16.49, n = 25). Data had Gaussian distribution, with low variability. Random effect 'hive' was not significant ($F_{4,20} = 2.09$, $p = 0.120$).

Expected CO_2 gradient across the cappings

Knowing the cappings conductance and the pupae respiration rate we aimed to determine the CO_2 concentrations inside the worker brood cell where honeybee worker pupae develop. In order to do so we first needed to measure the median area of the cell cappings that is 0.207 cm^2 (n = 18, Figure 5B). The CO_2 concentration inside the brood cell depends on CO_2 concentration in hive and the CO_2 gradient (that is the difference between the CO_2 concentration in the hive and in the brood cell) is calculated as a function of pupae respiration rate, capping area and capping diffusive conductance for CO_2. It will be constant if these parameters do not change. The extremes of respiration rates and capping diffusive conductance, in connection with almost invariable cappings area should define the span of the CO_2 gradient. According to our measurements, the resulting CO_2 gradient across the capping should be in a narrow range of 0.01% in case of minimal respiration rate and maximal capping conductance and 0.06% in the opposite contrast (0.03% using both median and average values). In other words, the median or average concentration of CO_2 in the brood cell will only be 300 ppm (0.03%) higher than the CO_2 concentrations inside the hive. The brood cappings allow very good exchange of CO_2 gasses and the CO_2 concentrations inside the brood cell will never exceed dramatically the CO_2 concentrations in the hive environment (Figure 5C).

On the other hand, the calculated CO_2 gradient for a hypothetical situation when the brood would be sealed with cappings from the honey cells lies in a broad range between 0.5 and 15.16% (1.47% in median, 2.81% in average) based on our experimentally determined values but in real situation it could be even higher, see discussion.

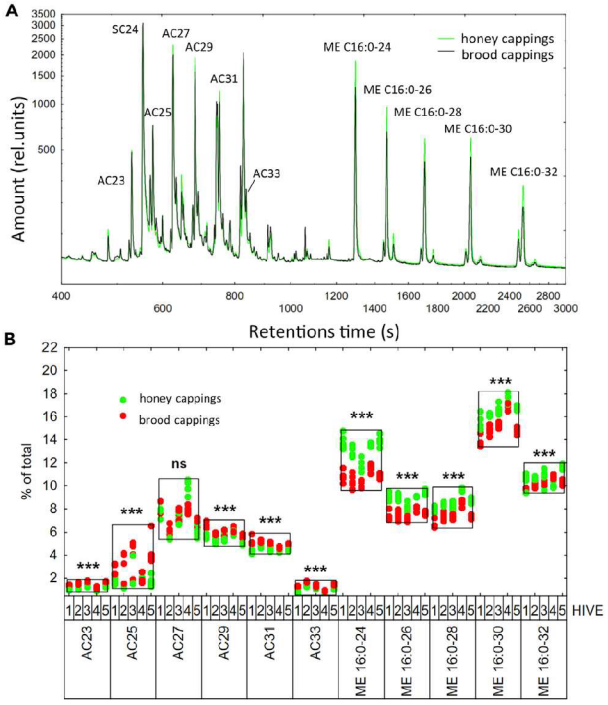


Figure 2. The n-alkane and monoester composition of the cappings

Five honey cappings and 5 worker brood cappings each from 5 different hives were dissected from the same combs and their composition assessed by GC-MS.

(A) Representative GC-MS chromatograms with individual peaks for n-alkanes (AC), monoesters (ME) and a standard C24 n-alkane (SC24).

(B) Quantification of peak sizes for individual n-alkanes and monoesters in honey and brood cappings. N = 25 for each type of cappings. Brood cappings contained lower percentage of monoester content compensated by higher percentage of n-alkanes when compared to the honey cappings (two-way ANOVA $F_{1,\ 4} = 123.8$, $p < 0.001$). The hives did not differ significantly when tested as a random factor ($F_{4,\ 23.7} = 2.20$, $p = 0.100$). ns indicates $p > 0.05$, ***$p < 0.001$.

CO_2 diffusive conductance of bumble bee cocoons and respiration of their pupae

To see the evolutionary conservation of the differential use of wax in the *Hymenoptera* family we compared the characteristics of the honeybee brood cell with the same parameters for the bumblee brood cocoons of *Bombus terretris*.

The cocoon consists of a bottom waxy part and an upper part that contains much less wax (Figure S1A). Inside of the cocoon there are silk fibers spun by the larva before pupation, similarly as in honeybees. The waxy bottom part of the cocoon was nearly impermeable, with the conductance of 0.06–1.5 mmol m^{-2} s^{-1} (median 0.49 mmol m^{-2} s^{-1}, n = 10, whereas the upper part was porous with conductance of 2.68–12.72 mmol m^{-2} s^{-1}(median 7.4 mmol m^{-2} s^{-1}, n = 10). The CO_2 diffusibility of the upper part of the cocoon was therefore 14.3-fold higher in median values than the CO_2 diffusibility of the bottom part (U = 0, Z = −3.74, $p < 0.001$) (Figure S1E).

Although the bottom part of the cocoon appeared as a continuous layer of wax (Figure S1B) the upper part contained areas that were porous and transmitted light under the microscope. They did not contain pores of defined shape but they resembled a net where the wax layer was very thin or missing in certain areas (Figures S1C and S1D).

The respiration of the bumble bee pupae at the white eye stage was higher than in honeybees (median 7.68 μmol h^{-1} $larva^{-1}$, n = 10) but that is not surprising given the bigger weight of the pupae (fresh weight median 0.345 g). When normalized to weight the respiration rate was remarkably similar (median 22.81 μmol g^{-1}FW h^{-1}, see Figure S1F and summary in Table S1). The area of the porous part

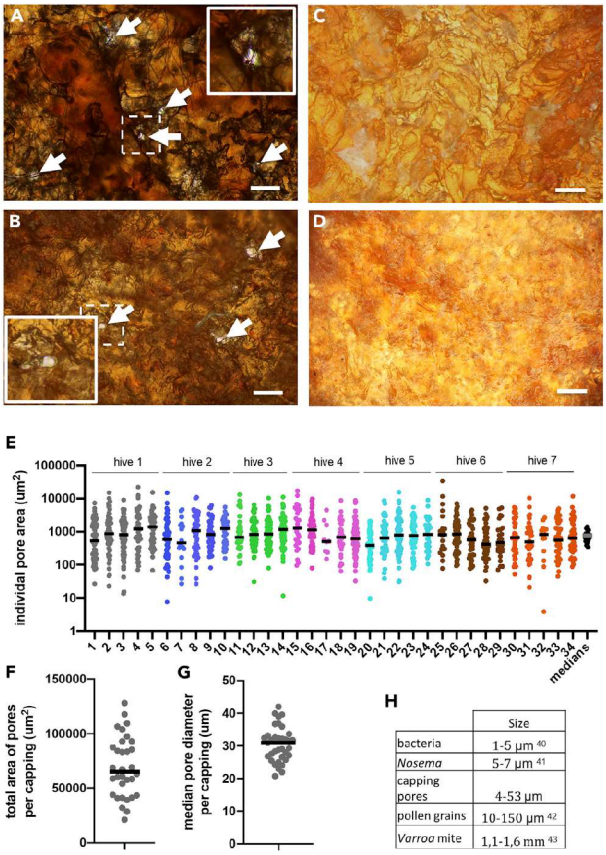


Figure 3. The pores in the worker brood cappings and their quantification

Microphotography of (A) inner and (B) outer surface of worker brood cappings and (C) inner and (D) outer surface of honey cappings. Arrows indicates pores throughout the brood cappings. Note the cocoon fibers going across the inner surface in A. Scale bar 100 µm. Insets in A and B show magnified regions indicated by dotted line in the main photograph.

(E) Quantification of the pores area per individual cappings across 7 different hives (5 cappings per hive) with median values. Individual values with medians.

(F) Quantification of the total area of all pores per capping (pooled data from 7 hives, n = 35 cappings). Individual values with median.

(G and H) Quantification of the median pore diameter (pooled data from 7 hives, n = 35 cappings) and (H) its comparison to the sizes of common pathogens and pollen grains found in the hive. See also Figure S1.

of the bumble bee brood cocoon was larger in comparison to the area of the honeybee brood capping (Figure S1G) but its conductance was smaller. The calculated CO_2 gradient across the cocoons made entirely from the porous material is 0.09%, similar to the 0.03% across honeybee brood cappings (Figure 5H). If the cocoon was made entirely from the compact waxy part the CO_2 levels inside it could reach up to 11% (in case of calculating with maximal pupa respiration and minimal cocoon conductance values we recorded).

DISCUSSION

Different cappings for different purposes

Bees have adapted to use wax in two contrasting biological ways: either as a nearly impermeable material insulating from the outside environment or as a permeable, porous material allowing the respiration and communication of developing pupa with its environment.

Similar but distinct evolutionary adaptations can be found in the wax usage of different *Hymenoptera*. While all the worker, drone and queen brood cappings of *A. mellifera* are intact, the drone brood cappings of *Apis cerana* and *Apis koschevnikovi* have a characteristic central opening.[27,28] These openings serves for

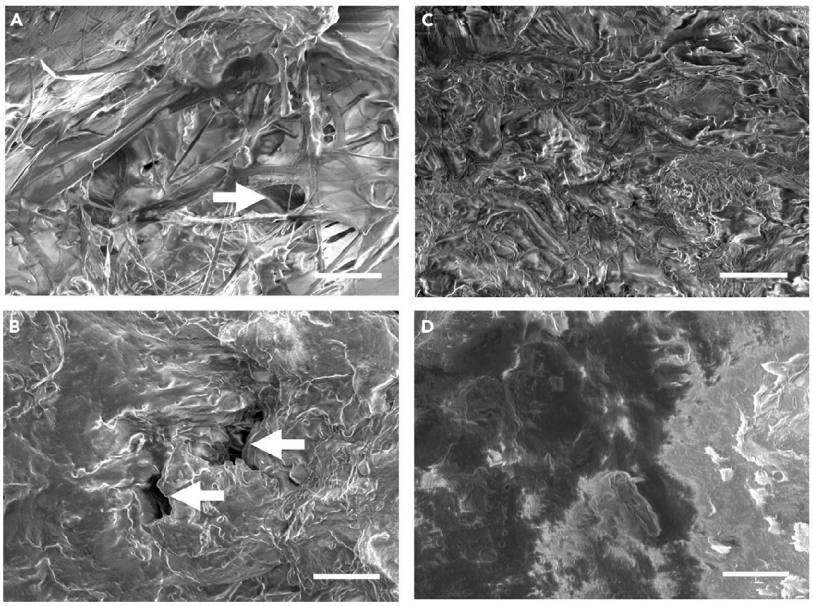


Figure 4. EM pictures of the pores in the worker brood cappings

(A–D) Scanning electron microscopy pictures of (A) inner surface of worker brood capping, (B) outer surface of brood capping, (C) inner surface of honey capping, and (D) outer surface of honey capping. Arrows indicate pores in the brood cappings, absent in the honey cappings. Scale bar 50 μm.

the exchange of respiratory gases by the developing drone pupae, as plugging them with beeswax leads to delayed metamorphosis or death.[29] Moreover, the central openings allow the workers to smell easily any volatile signals emanating from the pupa. When a pupa is infected with the parasitic *Varroa* mite or by viruses, volatile signals instruct the worker bees to close the central opening in the capping by wax, entombing and killing both the pupa and the mite within the cell.[30] Such hygienic behavior not only contributes to the varroa resistance of these species but it also puts a strong selective pressure on the mite to minimize the harm to the developing drone pupa.

In bumble bee colonies, the worker pupae develop in closed cells where the bottom part is made of wax but the upper part partially exposes the silky cocoon. Completely exposed cocoons are visible in the colonies of other social insect like wasps or ants (that neither raise brood nor store liquid food in wax cell-like structures within their colonies). Our data of bumble bee respiration and cocoon conductance suggest that the thin, porous layer of wax in the upper part of the cocoon allows sufficient gas exchange for the developing pupa. We conclude that bumble bees, similarly to honeybees, were able to evolutionary adapt to overcome the problem of low gas permeability of their waxy brood cells made of wax, by allowing some parts of it to be porous.

The honeybee brood cappings or the porous parts of the bumble bee cocoon represent a barrier against larger particles like grains of pollen that could potentially be a source of microbial contamination. However, despite the fact that the pores are large enough to allow entrance of individual bacteria or unicellular eukaryotes, it is unlikely that this way of entrance will play a major role in transmitting diseases. From the evolutionary point, we could speculate that capping of brood with wax was originally also beneficial by protecting the pupa from an attack of parasitic wasps, because it is common in some solitary bees such as the alpha-alpha leafcutting bees.[31] It is obvious that protection against parasitic wasps does not play a role in the socially advanced honeybee or bumble bee colonies but this aspect might have been important at the beginning of eusociality evolution when colonies were small and not tightly organized. In relation to honeybees, the capping does not prevent the brood from being infected by the parasitic varroa mite, because the mite enters the cell before it gets capped. Nevertheless, it might be interesting to determine if the structure of the cappings, their variations in porosity, together with the accompanying changes in gas permeability affects mites physiological parameters, for example its fertility.

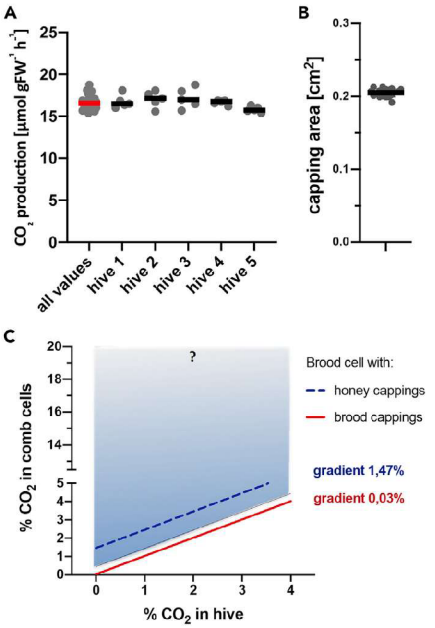


Figure 5. Respiration rate of honeybee pupae, capping area and the expected CO_2 concentrations in the wax cells with various cappings

(A) CO_2 production (respiration) of honeybee worker pupa, age 10 days since hatching (4 days after cell capping) in 5 different hives, 5 pupae per hive. Individual values with medians. There is no significant difference between the hives (two-way ANOVA $F_{4,20} = 2.09$, $p = 0.120$).

(B) Capping area of worker brood cell measured by optical microphotography. Individual values with medians (n = 18).

(C) The expected CO_2 gradient of worker brood capping and the expected physiological CO_2 concentrations in worker brood (red) and CO_2 gradient and concentrations in a hypothetical situation when worker brood would be covered with honey capping (blue). Blue rectangle shows the span of values for honey cappings based on minimal conductance and maximal pupa respiration and vice versa. The maximal span values of honey capping are likely even bigger, see discussion. On the contrary, the span for brood cappings is very narrow and invisible in the graph. See also Figure S1 and Table S1.

The brood cappings have substantially increased gas permeability – Evolutionary convergences and physiological implications

We are the first to quantify both the honey and brood cappings CO_2 conductances using a specialized tandem setup of Li-6400XT infra-red gas analyzers. We also used this up-to-date technique to measure the respiration of honeybee pupae and found remarkably similar values to the pupal respiration reported more than 80 years ago by *Melampy (1939)* using the Barcroft-Warburg manometric method[32] (Table S1).

The pores of µm sizes that we identified in brood cell capping are responsible for the large increase in gas permeability of brood cappings in comparison to the solid wax of honey storage cappings. The pores in the brood cappings thus allow efficient exchange of volatile compounds, such as oxygen, carbon dioxide, pheromones or kairomones. As we demonstrate the CO_2 concentration in the brood cells only mildly exceeds the CO_2 concentrations in the hive (gradient of only 0.03%, so 300 ppm difference). This is important for proper brood development but also for the colony's response to the pathogen infection.

It is remarkable that the conductance of the brood cappings for CO_2 resembles the CO_2 conductance of living cells, organs or surfaces, such as rat cardiomyocytes[33] or human erythrocytes,[34] despite being built from wax. The honey cappings have conductance on the opposite end of the biologically relevant spectrum, resembling the gas impermeable apical membrane of the gut cells[35] (see also Figure 1C).

Another notable example of a convergent evolution can be seen in the plant aerial surfaces that are also covered with waxy cuticle. The cuticle is impermeable for gases and its conductance is close to values we measured for honey cell cappings.[36] On the other hand, plants are able to substantially increase their conductance by means of closable pores – stomata. Strikingly, the percentage of brood capping area containing pores is similar to percentage of leaf area containing stomata.[37] From this respect it may not be surprising that stomatous plant surfaces, such as the lower leaf sides, have similar conductances to the brood cappings.[38]

Although the CO_2 conductance that we measured in the brood cappings is primarily the consequence of the pores, the low but not zero conductance of the honey capping is probably due to gas diffusion through the wax itself because of its partially crystalline structure[39] or to an occasional pores that we rarely observed. It may be an interesting future challenge to separate CO_2 flux into solid diffusion (wax) and air pathways using HelOx/NitOx techniques.[40]

Estimated CO_2 gradients and its consequences for brood development

Although it is obvious from our data that the brood cappings are well permeable to gases the honey cappings show very low and largely variable conductance. The conductivity data for honey cappings that we detected with the sensitive gas analyzers were close to the detection limit of the system and gave quite a wide range of small values close to zero, in a non-Gaussian distribution. Of interest, similar distribution of diffusive conductance was observed for intact plant cuticles.[41] In addition, we found that the honey cappings were sensitive to handling. Some of the honey capping had unexpectedly high conductivity but inspection under a stereomicroscope revealed cracks that were obviously not native but rather caused by manipulation. Such obvious outliers were excluded from the analysis but cappings with less apparent faults might have been still included. It is therefore possible that despite the fact that the median conductance value was calculated to 3.3 mmol m^{-2} s^{-1}, the real conductance of the honey cappings is lower, very close to zero. Such values would make the cappings virtually impermeable and the estimated CO_2 gradient in a speculative situation of brood covered by honey cappings would far exceed the 15.2% maximal values we report.

Therefore, using honey cappings to cover brood would be very risky for the honeybees, as their workers are obviously not able to control the conductivity of this type of cappings and the CO_2 levels in the brood cell could often rise beyond tolerable levels. Porous brood cappings, instead, seem to be perfectly elaborated and their conductance is more than sufficient and not deviating dramatically from Gaussianity.

Honeybees can tolerate a wide range of CO_2 concentrations and the CO_2 levels in the hive are maintained well above the 0.04% found in the free atmosphere. The usual CO_2 concentrations in a beehive in summer fluctuate between 0.1 and 0.3%[42,43] but up to 4% CO_2 has been measured in small experimental hives in laboratory settings.[44,45] Based on our data, the brood cappings are highly penetrable to CO_2. Consequently, the CO_2 concentration in a brood cell in the hive should lie within a narrow range of 0.13–0.33% for the majority of the season (Figure 5C).

Although the data on long term bee brood survival and physiological characteristics under elevated CO_2 are missing in the literature, it is well documented that high CO_2 exposure affects adult honeybee lifespan and interferes with their physiological functions.[46–48] From this respect it is not surprising that honeybees are able to sense CO_2 and its elevated levels and induce a fanning behavior to keep hive CO_2 concentrations within tolerable limits.[44]

We used the *A. mellifera carnica* worker brood cappings and honey cappings to measure their chemical, physical and structural properties and found them similar to the properties of the bumble bee pupae cocoons of *Bombus terrestris*. It is therefore likely that similar principles apply to other *Hymenoptera* that use sealed cells for brood rearing or for storing their reserves. Sealing the wax cells brings an evolutionary advantage for these species and their ability to create wax structures with contrasting permeabilities represents an elegant evolutionary adaptation.

Contrasting permeability of cappings as an evolutionary adaptation for using wax for nest building

When insects opted to use wax to cover their brood, they simultaneously needed to solve the issue of required gas permeability. However, whether this represented a real selection pressure or was merely reflected in the imperfect construction of such cappings (*i.e.* incorporating gas permeable pores or bare patches by default) is difficult to determine. Expressed another way, is it the cappings of brood or honey cells that represent the evolutionary adaptation? It is possible that initial imperfect cappings construction represented a default primary state that suited the respiratory requirements of covered brood, without the need of a selection. Accordingly, only later in their evolution did honeybees adapt this process to make the comparatively less permeable honey cappings, that in turn conveyed the evolutionary advantage of a long-term honey storage. However, although it is clear honey storage is an evolutionarily more recent development of the wax-building insects (as apparent from the bumble bee colonies that cover brood in wax but do not cap honey for long-term storage), it is still not easy to unequivocally determine whether it was the need for brood cappings permeability or honey cappings impermeability that represented the main selection pressure in the differential utilization of wax for these two types of cappings.

Limitations of the study

A limitation of our study may be in our focus on *A. mellifera carnica* bees without consideration of the biological variability across honeybees in different environments, their subpopulations and geographical origins. Although similar values for capping conductance were measured across all the hives examined we cannot exclude the values may change or be specifically adapted by the bees during the worker brood developmental stages. Methodically, uncertainty in close-to-zero conductances of honey cappings is another limitation. Li-6400XT gas exchange system is very sensitive but developed to measure plant leaves of 2 × 3 cm (6 cm^2). Honey or brood cappings have surface area about 0.2 cm^2, compromising the absolute sensitivity. The positively skewed statistical distribution of honey capping conductance supports this idea. Moreover, the wax cappings are very sensitive to manipulation. Microscopic cracks appear easily, increasing gas permeability substantially. Minimal conductance of honey cappings may thus be even lower than we measured. This effect would further increase the differences between the honey and brood capping conductances and strengthen the significance of our study.

STAR★METHODS

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  - Measurement of the capping permeability to CO_2
  - Respiration rate measurement
  - Microphotography, capped cell areas
  - Scanning electron microscopy
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105445.

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AUTHOR CONTRIBUTIONS

J.K. designed methods, measured and processed data, and made statistics. K.S. participated in respiration and wax analysis experiments, F.P. provided electron microscopy pictures. A.B.K. designed the study and participated in the experimental work. A.B.K. and J.K. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Apis mellifera carnica colonies	Experimental apiary of the Biology Centre AS CR, Ceske Budejovice, Czech Republic (48°58′31.924″N, 14°26′44.671″E; 390 m)	N/A
Bombus terrestris colony	Agricultural Science company in Troubsko, Czech Republic	http://www.ceskycmelak.cz/
Chemicals, peptides, and recombinant proteins		
C24 alkane	Sigma Aldrich	https://www.sigmaaldrich.com/CZ/en/product/sigma/t4758
Software and algorithms		
ImageJ	Schneider et al.[49]	https://imagej.nih.gov/ij/
STATISTICA 12	TIBCO Software Inc, Palo Alto, CA, USA	https://www.tibco.com/resources/
Other		
Gas chromatograph, Trace 1310	Thermo, Bremen, Germany	https://www.thermofisher.com/cz/en/home.html
Isotope ratio mass spectrometer (IRMS), Delta V Advantage	Thermo, Bremen, Germany	https://www.thermofisher.com/cz/en/home.html
Chromatography capillary column LION LN-05 Sil-MS	Chromservis, Prague, Czech Republic	https://www.chromservis.eu/en/lion-ln-5-sil-ms-gc-column-30-m-0-25-mm-0-10-m
6400XT infra-red gas analyser	LiCor, Nebrasca, USA	https://www.licor.com/env/products/photosynthesis/LI-6400XT/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Alena Bruce Krejčí (abruce@prf.jcu.cz or akrejci@prf.jcu.cz) both addresses work.

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Colonies of *A. mellifera carnica* were kept at the experimental apiary of the Biology Center AS CR, Ceske Budejovice, Czech Republic (48°58′31.924″N, 14°26′44.671″E; 390 m) according to standard beekeeping protocol, including honey harvest in June and July, supplementary sugar feeding in July and August and varroa treatment with amitraz in the autumn and oxalic acid in winter. The levels of *Varroa destructor* were regularly monitored by alcohol washes and did not exceed 2% level during the whole season. Combs were built on wax foundations with 5.2 mm in short diagonal.

Bumble bee colony of *B. terrestris* was purchased in May 2022 from the company Agricultural Science in Troubsko, Czech Republic and placed at the same apiary as beehives described above.

METHOD DETAILS

GC/MS analysis of waxes

Cappings were dissolved in chloroform (1 mg mL^{-1}) and 2 μL of the sample were injected in split/splitless injector at 300°C of the GC (Trace 1310, Thermo, Bremen, Germany). Injection was splitless for 1.5 min, then split with flow of 100 mL per minute for the next 1 min, and 5 mL per minute (gas saver) for the rest of analysis. Fast column LION LN-05 Sil-MS (30 m × 0.25 mm x 0.1 μm film thickness) was used with helium flow rate 1.5 mL per minute. The oven temperature program was set to 50°C during injection and for next 2 min, then increasing to 200°C (slope 40°C/min), further to 320°C (4°C/min) and isothermal at 320°C for the rest of the analysis (*ca* 60 min in total). Eluting compounds were oxidized to CO_2 via IsoLink II interphase (Thermo, Bremen, Germany) at 1000°C and introduced to continuous-flow isotope ratio MS (Delta V Advantage, Thermo, Bremen, Germany). Internal standard n-tetracosane (C24 alkane) was added to the samples in concentration of 20 μg mL^{-1} to quantify compounds. GC calibration curve for alkanes C10 to C40 was created to correct for any GC sensitivity drops for the high-boiling compounds. Principal compounds (alkanes and wax esters) were identified via available standards and/or literature available wax chromatograms.[11,12,21–24] We also measured subset of derivatized samples (50 μL of BSTFA +100 μL of pyridine, 2 h at 80°C[25]) and found that overall peak area did not differ substantially (data not shown). The cappings also contain chloroform insoluble substances such as silk, pollen or hive debris but these were not quantified in our study. Five brood cappings and five honey cappings from the same comb were analyzed for 5 different honeybee colonies (n = 25 for brood cappings and n = 25 for honey cappings).

Measurement of the capping permeability to CO_2

The honeybee brood cappings and honey cappings were carefully dissected from individual combs using scalpel and a pair of fine forceps in June 2022. Five cappings of both types were measured for each of 5 different hives (n = 25 for brood cappings and n = 25 for honey cappings). The CO_2 diffusive conductance of the cappings was measured by a unique experimental system where two LI-6400XT units assembled together with their leaf chambers complemented in 'inverse' position (Figure 1B). One unit contained only the bottom part of chamber while the other unit contained only the upper part of chamber with an LED light source (not applicable here). A plexiglas plate 2 mm thick with a central hole of 3 mm in diameter was inserted between the chambers where plant leaf would normally be positioned. Dissected honey- or brood capping was sealed in the hole by pressing gently on its edge. The CO_2 concentration in the upper unit was set to 2300 μmol mol^{-1} (maximal available concentration of the instrument) whereas lower unit was scrubbed of all CO_2, producing a CO_2 free air. The 'matching conduct' commonly present in the bottom part of the chamber was replaced with a needle and tubing. the setup was already optimized for measurement of amphistomatous leaves, where non only diffusional but also as bulk flow may occur across the leaf (stoma – intercellular air – stoma continuum). Accordingly, it was possible to monitor and maintain the pressure differences between the adaxial and abaxial unit less than 0.1 mBar (10 Pa) that is important to prevent a bulk flow across the pores.

The bumble bee brood cocoons were collected in June 2022 from a single colony of *B. terrestris*. Ten cocoons with white eyed pupae were carefully cut in their equatorial position and the upper (porous) and bottom (waxy) parts were measured separately using the tandem LI-6400XT system described above, with a thin parafin seal between the cocoon and the plexiglas in the measuring chamber.

Respiration rate measurement

Respiration of healthy honeybee worker brood pupae originating from 5 different colonies was examined at the stage of white to pink eyed pupae, 4 days post capping (10 days old since hatching). Five pupae were measured from each hive (n = 25). The bumble bee pupae of the white eye stage originated from a single colony of *B. terrestris*. The bumble bee brood respiration was measured in 10 intact cocoons and the stage of brood development was only examined after the cocoon dissection at the end of the experiment.

The rate of CO_2 production of individual pupae was measured by Li-6400XT infra-red gas analyser with insect respiration chamber. The cappings of the brood cells were removed, respiring pupae were gently pulled out with entomological tweezers and maintained at 35°C throughout the measurements. The fresh

weight (FW) and the dry weight (DW) of pupae was determined using microscales. Single pupae was inserted in the insect respiration chamber continuosly flushed with Li-6400XT sample CO_2 free air stream of 150 μmol air s^{-1}. After 5 min of stabilization, data were recorded for 3 min. We measured the average CO_2 increase about 5 μmol mol^{-1}, while the system noise was lower than 0.1 μmol mol^{-1}, allowing us to determine the respiration rate with sufficient precision. As control, empty chamber was also measured regularly and any offset subtracted. The respiration rates were normalized both to FW or DW (see Table S1).

Microphotography, capped cell areas

Optical images of wax cappings were taken using Olympus BX61 microscope with a combination of transmitted and reflected light (to maximize pore to solid wax contrast) using objectives of 4x (frame 5.5 mm wide) and 10x (frame 2.2 mm wide). Sets of sequential images with focus stacking were merged into one picture with extended depth of field using the Combine ZP software. To precisely calculate the average area of cappings, individual unsealed honey comb cells were photographed (n = 18) under a stereomicroscope with a reference scale and their inner area calculated with ImageJ Software.[26] Individual halves of the bumble bee cocoons (n = 11) were also photographed and equatorial diameter was used to calculate surface area approximated as a hemisphere + cylinder 3 mm in height.

Individual pore area, total pore area per brood capping and median pore diameter were quantified in ImageJ using microphotographs of cappings originating from 7 different hives, with 5 cappings measured for each hive (n = 35).

Scanning electron microscopy

For scanning electron microscopy (SEM), samples were coated by gold (2 nm thin layer) in an ion sputter coater (Bal-Tec SCD 050) and observed with a JEOL JSM-IT 200 microscope.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistics was performed in the *Statistica 12* software (TIBCO Software Inc, Palo Alto, CA, USA). Wax composition (Figure 2B) and bee-pupae respiration (Figure 5A) had a Gaussian within-group distributions (Shapiro-Willk test) and therefore they were tested for differences using ANOVA. Wax composition was tested by 3-way full factorial ANOVA(fixed factors: 'compound', 'capping type'; random factor: 'hive'). Pupae respiration rates were tested by 1-way ANOVA(factor: 'hive'). Capping diffusive conductances (Figures 1A and S1), in turn, had positively skewed distribution, mainly in group of 'honey cappings', and therfore they were tested by Mann-Whitney U test (factor: 'capping type'). Graphically, raw data distributions, rather than descriptive statistics, are presented. Significance was defined as $p < 0.001$ (***), $0.001 < p < 0.01$(**), $0.01 < p < 0.05$ (*), $p > 0.05$ (ns). The sampling and number of replicates are indicated in the specific sections of the STAR methods and in the figure legends.

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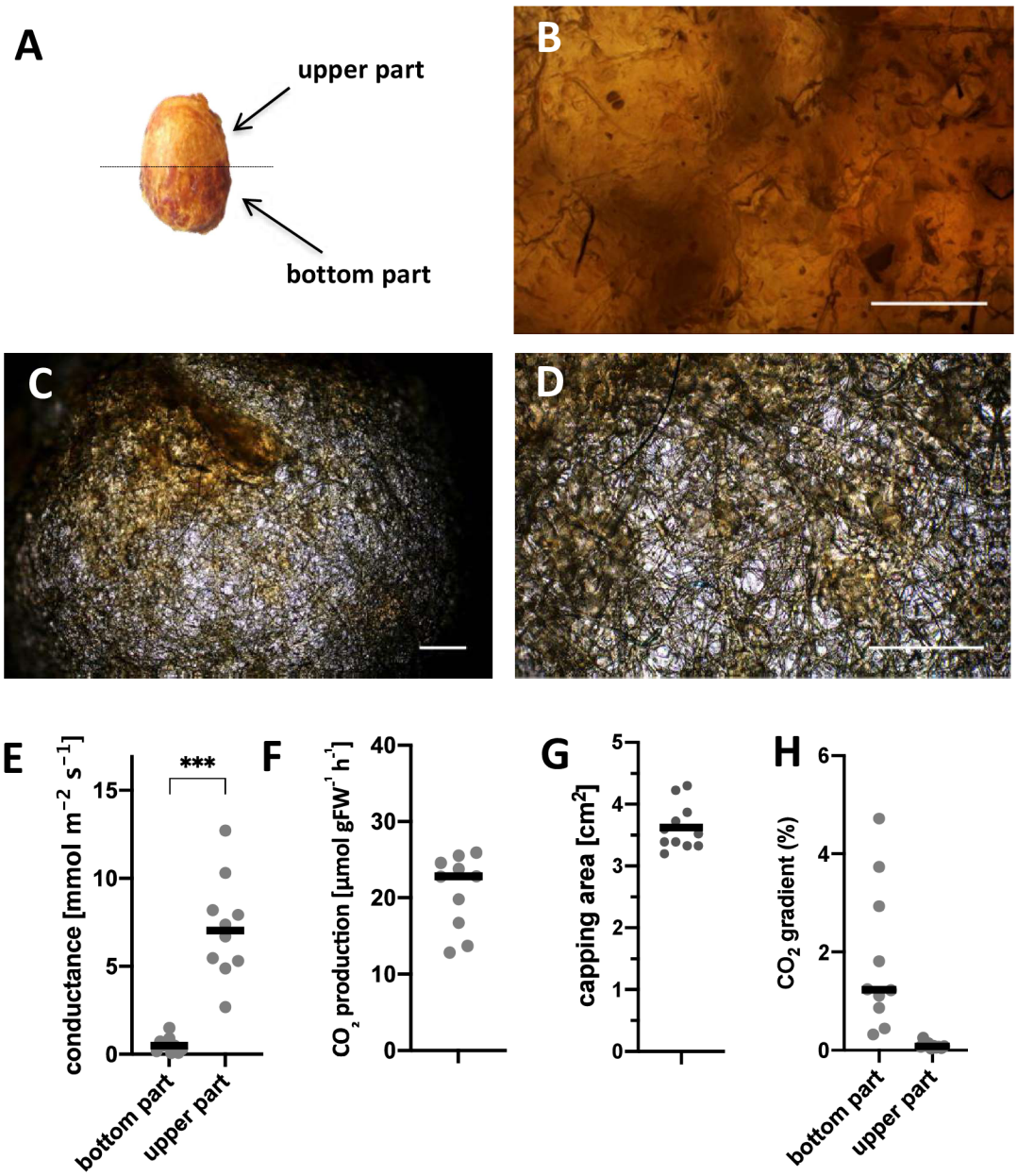
Supplemental information

Honeybees control the gas permeability of brood and honey cappings

Jiří Kubásek, Karolína Svobodová, František Půta, and Alena Bruce Krejčí



Suppelmentary figure S1: The conductace of bumble bee brood cocoons (Bombus terrestris), respiration of their pupae and expected CO_2 gradient across the cocoon. Related to Fig. 1, 3 and 5. (A) The cocoon consists of bottom waxy part and upper porous part. (B) Microphotography of the bottom waxy part. Scale bar 500 µ m. (C, D) Microphotography of the upper porous part. Scale bar 500 µ m. (E) The conductance of the upper and bottom part of the cocoon (n=10). Mann-Whitney U test (U=0, Z= -3.74, p<0.001) (F) CO2 production of the white eyed pupae (n=10). (G) The area of the porous half of the cocoon (n=10). (H) Calculated CO2 gradients across the cocoons in case they were made entirely from the material forming the bottom or upper part (n=10). E-F: Individual values with median. See also Supplementary table S1.

	Age of pupae	Method	CO2 produced	CO2 produced	fresh weight	respiration rate FW	respiration rate FW	dry weight	respiration rate DW	respiration rate DW
	days		(µmol h-1 per pupa)	(mm-3 h-1 per pupa)	(g)	(µmol gWW-1 h-1)	(mm-3 gFW-1 h -1)	(g)	(µmol gDW-1 h-1)	(mm-3 gDW-1 h -1)
Apis mellifera (this study)	10	Li-6400XT	2.37	53.02	0.1445	16.39	367.14	0.0296	79.92	1790.21
Apis mellifera (Melampy,1939)	10	Barcroft-Warburg	2.42	54.1	0.1356	17.81	399.00	-	-	-
Bombus terrestris (this study)	-	Li-6400XT	7.13	159.63	0.3479	20.85	533.08	0.10103	71.37	1767.93

Supplementary table S1: The overview of values for honey bee and bumble bee pupae weight and respiration. Relates to Fig. 5 and S1.

DISCUSSION

Massive colony losses of *A. mellifera* appearing in recent decades have sparked significant efforts among scientists and beekeepers to diminish the detrimental effect of varroa mite, viruses and other pathogens. Current strategies to prevent these losses primarily focus on the elimination of varroa that is considered the main driver of colony decline due to its role in transmitting and amplifying viral infections. However, it is the viral infections rather than the mites themselves, that are the principal cause of colony mortality. Greater attention should therefore be directed toward developing approaches that target the spread of viral diseases. The work present in this thesis aims to test solutions targeting honey bee viruses, and to explore factors connected with varroa resistance.

Chapter I of this thesis aimed to test the antiviral potential of the gypsy mushroom (*Cortinarius caperatus*) against DWV. Currently, only a limited number of compounds are being tested for antiviral efficacy against honey bee viruses (Pascual et al., 2023; Felicioli et al., 2020; Stamets et al., 2018, Desai et al., 2012) and no treatments specifically targeting these viruses are commercially available. In this project, we expanded the range of affordable and widely accessible compounds that could serve as antiviral treatments for DWV, as demonstrated in both cage and hive experiments. Importantly, the gypsy mushroom treatment had no adverse effects on the lifespan of caged honey bees and left no detectable mushroom residues in honey.

Our results showed that honey bees fed with 1% alcohol extract of gypsy mushroom did not experience the progression of DWV infection as seen in control honey bees. Instead, the viral loads in the treated honey bees remained unchanged across the experiments. The mechanism of action of the mushroom extract was not determined within this project. However, alongside the inhibition of DWV progression, these honey bees showed increased expression of Tep7, an immune effector protein similar to mammalian complement (Shokal & Eleftherianos, 2017), and Bap1, which has antiviral effects, though its regulation is unknown (McMenamin et al., 2021). They also exhibited lower transcription of Vago, an antiviral immune modulator (Niu et al., 2016). These immune gene transcriptional changes may emerge from either direct viral inhibition or immune stimulation by compounds in the mushroom extract. For instance, these compounds might inhibit viral replication by binding to key viral enzymes, as observed in influenza virus treated by *Phellinus linteus* (Hwang et al., 2018) and HIV treated by *Ganoderma lucidum* (El-Mekkawy et al., 1998) or lactose of *Lentinus tigrinus* (Xu et al., 2012). Given that

viruses can modulate host immune responses (Alcami et al., 2002), it is plausible that DWV suppresses specific immune effectors to evade host defenses. Consequently, the higher levels of Tep7 and Bap1 in mushroom-treated honey bees with lower DWV titers, compared to controls, could reflect reduced immunosuppression caused by DWV. The lower transcription of Vago also aligns with lower DWV loads in mushroom-treated honey bees, similarly as observed by Niu et al. (2016). Alternatively, compounds in the mushroom, such as beta-glucans (Guluarte et al., 2023), may induce immune responses, thus act indirectly against the progression of DWV infection. However, since beta-glucans are insoluble in alcohol, they are unlikely to be responsible for the antiviral effects observed in this study.

Although 1% alcohol extract of gypsy mushroom inhibits the progression of DWV infection, we also noticed that this effect depends on the initial levels of infection. Specifically, the treatment of caged honey bees with DWV levels exceeding 10^8 copies per bee did not affect the progression of the infection (unpublished data). This observation indicates that the mushroom extract is likely ineffective against progressed DWV infection and could rather serve as prevention before reaching detrimental levels.

The application of 1% gypsy mushroom extract to hives through supplementary sugar feeding in late summer also inhibited DWV progression for 14 days. The quantification of varroa level infestation in experimental colonies excludes the possibility that the observed effect is attributed to unequal distribution of varroa mite infestation across the colonies. However, the mushroom extract treatment resulted in only about 10-fold lower DWV loads compared to controls, which is, though significant, a relatively minor difference. We believe that it is likely caused by a slow progression of the infection in control colonies. Although sharp increases in DWV loads alongside varroa infestation levels usually occur in the late summer season, the year of the experiment was unexpectedly mild in this term. It is also possible that higher concentration of the mushroom extract would lead to a more significant impact on the DWV suppression. The effect of gypsy mushroom in seasons characterized by significant risks of viral infections and parasite infestations deserves further investigation.

Chapter II examines the differences in the assembly of gut bacterial communities between Gotland varroa-surviving honey bees and local varroa-susceptible honey bees. It also explores the differential correlations between several honey bee pathogenic viruses and bacterial taxa within both honey bee groups. To achieve this, we conducted a bioinformatic analysis of a 16S rRNA dataset generated and published by Thaduri et al. (2021). To expand our understanding

of the bacterial communities in varroa-surviving honey bees, we used a network approach to examine co-occurrence patterns within the gut bacteria, as well as interactions between those bacteria and pathogenic honey bee viruses. We found that the co-occurrence networks of both honey bee groups were more similar than random in terms of closeness centrality and hub taxa, which corresponds with the fact that worker honey bees possess an effective and highly conserved core of symbiotic bacterial species, present in every worker western honey bee regardless of genetic background (Kwong & Moran, 2016).

However, the network of varroa-surviving honey bees was simpler, with fewer correlating taxa and more competitive interactions compared to the bacterial network of varroa-susceptible honey bees, which exhibited about three times more positive correlations and fewer negative associations. This result could relate to the findings of Thaduri et al. (2021), who reported significantly lower evenness and Shannon index values in varroa-surviving honey bees compared to varroa-susceptible ones, with the differences attributed mainly to minor opportunistic bacterial taxa. These results suggest that the microbiota of varroa-surviving honey bees may be more effective at controlling the growth of opportunistic bacteria and potentially prevent gut dysbiosis. However, as Thaduri et al. (2021) suggested, the observed differences could also be due to varroa-surviving honey bees having smaller colonies, less brood, and more frequent swarming compared to varroa-susceptible honey bees. It could affect the division of labor among workers (Johnson, 2010), which is an important factor in shaping the bacterial community (Jones et al., 2018; Baud et al., 2023). As a result, even though the honey bees were staged to be of identical age, they may be at different phenotypic stages, potentially biasing the interpretation of these results.

The inclusion of five pathogenic viruses in the bacterial networks revealed that the abundance of bacteria in varroa-surviving honey is less correlated with the loads of these viruses than the bacteria in varroa-susceptible honey bees. In varroa-surviving honey bees, *Lactobacillus* and *Bifidobacterium* correlated with BQCV and LSV, while in varroa-susceptible honey bees, *Lactobacillus*, *Bifidobacterium,* and *Gilliamella* had strong correlations with ARV-1, BQCV, LSV, and SBV. Because levels of these viruses were similar in both groups of the varroa-surviving honey bees, this result may indicate that the bacterial community of varroa-surviving honey bees is less affected by viral impacts, such as induction of immune responses. However, due to the lack of virus-free colonies in the study, we do not have reliable controls to see the impact of the viruses on the microbiome of both honey bee groups. In addition, it is surprising

that all correlations between bacteria and viruses are positive. Since virus-host-bacteria interactions are highly complex and our bioinformatic findings are not experimentally validated, we can only speculate on the possible causes of these observations. Although the relationships between honey bee pathogenic viruses and gut bacteria remain largely unexplored, Liu et al. (2024) showed that experimental infection of honey bees with IAPV led to a reduction in gut bacterial taxa. This reduction likely resulted from IAPV-induced immune responses also affecting gut bacteria (Deng et al., 2022). Therefore, one would expect the observation of negative correlations between the viral loads and gut bacteria abundance, yet it was not our case. The majority of viruses included in our analysis, such as LSV, BQCV, ARV, and their effects on the immunity and physiology of adult worker honey bees, are scarcely studied; moreover, honey bee pathogenic viruses also interact with each other, some of them synergistically, others antagonistically (Durand et al., 2023). Therefore, we cannot rule out the possibility that complex and unexplored virus-host-bacteria interactions may have shifted the microbial balance in favor of *Lactobacillus*, *Bifidobacterium*, and, in the case of varroa-susceptible bees, *Gilliamella*, without negative effects on other microbial community members, as seen in the absence of negative correlations between viruses and other bacterial taxa.

However, we speculate that, rather than indicating a causal relationship, the observed correlations may result from factors not considered in this study but similarly affecting bacteria and viruses. For example, the experimental honey bee colonies were not tested for the presence and abundance of microsporidia and trypanosomatids, such as *N. ceranae* and *L. passim*. The infection of *N. ceranae* reduces the efficacy of Imd and Toll signaling pathways (Antúnez et al., 2009; Li et al., 2018), which leads to the increased propagation of pathogens (Zheng et al., 2015) and gut symbionts (Lau et al., 2024). Thus, *N. ceranae* infection could hypothetically create the appearance of positive correlations between viruses and bacteria in our analysis, even if they had no impact on each other. Another possibility is the influence of external conditions affecting experimental colonies. The composition of honey bee microbiota changes across the season (Thaduri et al. 2021), influenced by varying foraging opportunities and food sources (Li et al., 2022). Similarly, certain viruses, such as LSV and SBV, also display seasonal fluctuations, peaking at the end of spring and reaching their lowest levels in the fall (Thaduri et al. 2021). Therefore, if nutritional opportunities promote the growth of certain bacteria during the periods of seasonal viral peaks, or conversely, if decreasing food sources at the end of the season lead to a decrease in some bacterial taxa coinciding with a decrease in viral abundance, we may observe these bacteria and viruses as having positive correlations even if the trends

would be only coincidental. Therefore, the elucidating of relationships between varroa resistance, pathogenic viruses, and gut bacterial communities requires further investigation.

Chapter III of this thesis investigates the differences in honey and brood wax cappings in terms of their structure, underlying chemical composition, and physical properties. By measuring the CO_2 conductance of both types of cappings and assessing the respiration of developing pupae, we were able to calculate CO_2 gradients across the cappings. While CO_2 dynamics in honey bees have been extensively studied, the CO_2 conductance through wax cappings was not known. Similarly, specific production of CO_2 by pupae has received limited attention, and knowledge of resulting gradients in sealed brood cells was lacking. The elucidating of these unknowns provides a fundamental base for our further research, in which we hypothesize that the changes in pupal respiration caused by varroa mite may possibly act as a signal contributing to the induction of hygienic behavior, alongside previously identified volatile compounds (Liendo et al., 2021).

Our results showing a significant difference in CO_2 conductance between the two types of cappings likely reflect their different biological purposes. The sealing of honey stores must be impermeable to humidity to protect honey from water reabsorption, and the nearly absent gas conductance of honey cappings observed in our study aligns well with this fact. In contrast, pupae present in sealed brood cells need to breathe. Additionally, the passage of volatiles produced by brood is extremely important in the context of hygienic behavior associated with varroa resistance, because specific volatiles produced by varroa-infested brood induce VSH behavior (Liendo et al., 2021). It is likely that specific volatile profiles associated with other pathogens and diseased brood also trigger hygienic behavior. As opposed to honey cappings, the measured values of CO_2 conductance in brood cappings indicate effective gas exchange. These differences are associated with the microstructures of the two types of cappings, because unlike honey cappings, which have a smooth and uniform surface, brood cappings are perforated with numerous micropores.

The respiration rates of honey bee larvae change significantly during development. As larvae grow, their overall CO_2 production consistently increases, but the respiration rate per unit of weight declines markedly by the sixth day after hatching (Melampy & Willis, 1939; Petz et al., 2004). This decline signals the onset of the pre-pupal stage. Melampy and Willis (1939) observed that honey bee respiration remains constant from the sixth to the twelfth day and then rises slightly until the imago emerges. Our measurements of the CO_2 respiration rate in 10-day-

old pupae (1.98–2.69 µmol/h/pupa) vary less than two-fold from the results of Melampy and Willis (1939), which are surprisingly similar even though we used different methodology and honey bees of different backgrounds. Nevertheless, like the results of Melampy and Willis (1939), our findings were obtained under laboratory conditions. Thus, variations in the real environment of a beehive can be possibly expected. For example, larval respiration rate is significantly associated with temperature (Petz et al., 2004), therefore, the suboptimal temperature conditions within a colony could result in varying respiration rates.

Based on the measurement of brood cappings gas conductance and pupal respiration rate, we determined that the general CO_2 gradient through the cappings of 10-day-old healthy pupae was 300 ppm (0.03%) higher than the CO_2 concentration inside the hive. This result is crucial for testing our hypothesis that changes in CO_2 gradients of varroa-infested pupae may act as a signal contributing to honey bee sensing of the infestation and subsequent induction of VSH. Nevertheless, the effect of varroa infestation on the respiration rates of honey bee pupae is not currently known. Active immune responses have high energy demands, triggering metabolic changes (Dolezal et al., 2019), which result in a decrease in respiration rates as shown in *Drosophila* infected with *Drosophila* C virus (Arnold et al., 2013). However, the effect of possible immune suppression induced by varroa parasitism (Gregory et al., 2005; Annoscia et al., 2019) combined with the injury caused by varroa feeding on a specific metabolism of pupae may not be as straightforward and requires further investigation. Any changes (positive or negative) in pupal respiration after pathogen exposure will lead to changes in CO_2 concentrations under the brood capping. If bees are able to sense these changes, the CO_2 level could serve as a universal indicator of the overall health status of the developing pupa and hence as a universal trigger of hygienic behavior. Future research needs to address the magnitude of the changes in CO_2 concentrations after pathogen exposure and the sensitivity of CO_2 detection by the honey bees.

Stange and Diesendorf (1973) showed that honey bee antennae sensilla have varying sensitivities to CO_2, with detection thresholds ranging from 10^{14} to 3×10^{17} molecules/ml. This suggests that 300 ppm CO_2 could potentially excite the most sensitive sensilla, and if varroa infestation increases the respiration rate of pupae, the resulting CO_2 gradient could be noticeable for honey bees. In a similar way, if pupal respiration decreases after varroa exposure, the negative change could still be detectable by the bees in comparison to the relatively high CO_2 background observed within the hive. However, the ability of honey bees to detect small

differences in CO_2 concentrations within a high CO_2 background is not known. As far as we know, the ability of honey bees to detect differences in CO_2 concentrations has only been assessed in a single study. This study demonstrated that honey bees can reliably distinguish the difference between airflows containing 1% and 3% CO_2, while the difference between 1% and 2% CO_2 was below the honey bee reaction threshold (Leher, 1966). It also showed that honey bees were more successful at detecting smaller differences in lower CO_2 concentrations; for instance, they could reliably recognize the difference between 1% and 3% CO_2 but could not detect the difference between 10% and 13% CO_2 (Leher, 1966). However, this ability has not been tested across diverse honeybee populations. Given the fact that varroa-resistant honey bees can recognize olfactory signals associated with varroa infestation better than nonresistant honey bees (Mondet et al., 2021), we speculate that they may be more sensitive also to changes in CO_2 levels. Moreover, it has been shown (Perez & Johnson, 2019) that only a small proportion (3–5%) of specialized bees within the colony are able to perform the hygienic behaviour, and we can assume that these bees may have much higher sensitivity to odor signals than other bees and than bees in the above-cited study.

CONCLUSIONS

This thesis explores factors influencing honey bee health from three different perspectives. Chapter I demonstrates that alcohol extracts from the gypsy mushroom can inhibit the progression of DWV infection in caged honey bees without having a negative effect on their lifespan. While this inhibitory effect is likely to also act in honey bee colonies, its antiviral potential needs validation in years of elevated honey bee mortality. Chapter II shows that the gut bacterial community of Gotland varroa-surviving honey bees appears more resilient, being less susceptible to invasion by opportunistic bacteria and the impact of pathogenic viruses compared to varroa-susceptible local bees. Chapter III shows that because of their microstructure, wax cappings differ in CO_2 conductance. Effective gas exchange through brood cappings, together with pupal respiration, creates a CO_2 gradient. Inferring this gradient lays the foundation for our future research on factors potentially contributing to the induction of hygienic behavior.

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Curriculum vitae

Education

2020 – now	**University of South Bohemia in České Budějovice** Faculty of Science Department of Molecular Biology and Genetics **PhD study: Integrative Biology**
2018 - 2020	**University of South Bohemia in České Budějovice** Faculty of Science Department of Molecular Biology and Genetics **Master study: Molecular and Cell Biology and Genetics**
2014 - 2018	**University of South Bohemia in České Budějovice** Faculty of Science Department of Medical Biology **Bachelor study: Laboratory and Medical biology**

Internships

11. 2022 and 3. + 4. 2024	**INRAE, French National Research Institute for Agriculture, Food and Environment, Maisons-Alfort, France** **Laboratory of Tick Microbiology (Dr. Alejandro Cabezas-Cruz)** Research activity within a scope of PhD internship Job description: Analysis of honeybee microbiota, writing scientific texts

Publications in Impact Journals

1. Svobodová, K., Krištůfek, V., Kubásek, J., & Bruce Krejčí, A. (2023). Alcohol extract of the gypsy mushroom Cortinarius caperatus inhibits the development of Deformed wing virus infection in western honey bee (Apis mellifera). *Journal of Insect Physiology*. https://doi.org/10.1016/j.jinsphys.2023.104583

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