Antimicrobial screening of crude extracts of *Talaromyces purpureogenus* strains (Ascomycota) from bee bread of honey bee *Apis mellifera*

by

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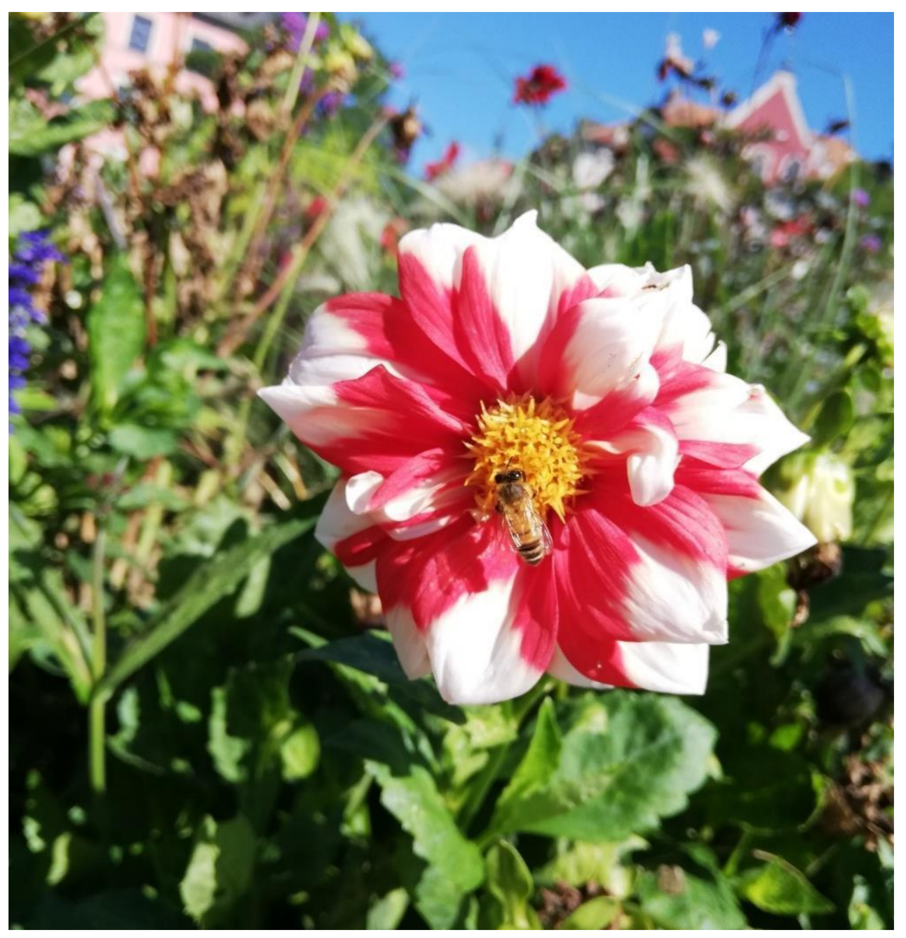

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Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and cited all text passages that are derived verbatim from or are based on the content of published work of others, and all information relating to verbal communications. I consent to the use of anti-plagiarism software to check my thesis. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University Giessen "Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis" in carrying out the investigations described in the dissertation."

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Summary

Shortfalls in the pollination service provided by wild and managed pollinators, such as honey bees *Apis mellifera*, threaten agricultural production and global food security. Although the use of therapeutics helps to control the *Varroa* mite infestations, and the pathogen load in *A. mellifera* colonies, more effective and sustainable strategies are needed to prevent the losses. Honey bees coexist with fungi that colonize hive surfaces and pollen, some of them are opportunistic pathogens, but many are beneficial species that produce antimicrobial compounds for pollen conservation and regulate pathogens. Herein, I describe seven fungal strains of *Talaromyces purpureogenus* from the bee bread of *A. mellifera* and investigate the antimicrobial potential of their crude organic extracts against honey bee pathogens under laboratory conditions. First, I added the extracts to a diet of bees infected with the chronic bee paralysis virus (CBPV). Then I tested the *in vitro* activity of the extracts against *Paenibacillus alvei* (associated with European foulbrood disease) and three *Aspergillus* species that cause stonebrood disease. The antiviral effect was further determined in mammalian cell lines against feline calicivirus (FCV), feline coronavirus (FCoV), and influenza viruses.

Three extracts (from strains B13, B18, and B30) mitigated CBPV infection and improved the survival rate of bees, whereas other extracts had no effect (B11 and B49) or were harmful (B69 and B195). In the mammalian cell lines, extract B18 inhibited the replication of FCV and FCoV in mammalian cells and reduced the infectivity of FCoV by ~99%. The extract B195 also reduced the FCoV infectivity (by ~90%) but caused cytotoxicity at higher concentrations, which could explain the negative effect on honey bee survival rate. The protective effect of the extracts B18 and B195 (at non-toxic concentrations) was also observed against influenza A viruses. In the antibacterial assay, the extracts B18 and B195 inhibited the growth of *P. alvei* at a concentration of 0.39 mg/mL. Bioactivity-guided dereplication revealed that the activity correlated with the presence of diketopiperazines, a siderophore, and three unknown compounds.

The results indicate that the compounds obtained by fermentation from *T. purpureogenus* extracts from are suitable as prophylactic or therapeutic feed additives to promote the resistance of honey bees to viral and bacterial pathogens. Furthermore, I propose that non-pathogenic fungi such as *Talaromyces* spp. and their metabolites in bee bread are an unexplored source of compounds that could be an important prerequisite for disease prevention. Agricultural practices that involve the application of fungicides can disrupt the fungal community and therefore negatively impact the health of bee colonies.

// Zusammenfassung

Ausfälle in der Bestäubungsleistung von wilden und bewirtschafteten Bestäubern, wie der Honigbiene *Apis mellifera*, bedrohen die landwirtschaftliche Produktion und die weltweite Ernährungssicherheit. Obwohl der Einsatz von Therapeutika hilft, den Befall mit der Varroa-Milbe und die Erregerlast in *A. mellifera*-Völkern zu kontrollieren, sind wirksamere und nachhaltigere Strategien erforderlich, um die Verluste zu verhindern. Honigbienen leben mit Pilzen zusammen, die Bienenstockoberflächen und Pollen besiedeln. Einige von ihnen sind opportunistische Krankheitserreger, aber viele sind nützliche Arten, die antimikrobielle Verbindungen zur Pollenkonservierung produzieren und Krankheitserreger regulieren. In diesem Artikel beschreibe ich sieben Pilzstämme von *Talaromyces purpureogenus* aus dem Bienenbrot von *A. mellifera* und untersuche das antimikrobielle Potenzial ihrer rohen organischen Extrakte gegen Krankheitserreger der Honigbiene unter Laborbedingungen. Zunächst habe ich die Extrakte zu einem Futter für Bienen gegeben, die mit dem Virus der chronischen Bienenlähmung (CBPV) infiziert waren. Dann testete ich die In-vitro-Aktivität der Extrakte gegen *Paenibacillus alvei* (der mit der Europäischen Faulbrutkrankheit in Verbindung gebracht wird) und drei Aspergillus-Arten, die die Steinbrutkrankheit verursachen. Die antivirale Wirkung wurde außerdem in Säugetierzelllinien gegen feline Caliciviren (FCV), feline Coronaviren (FCoV) und Influenzaviren bestimmt.

Drei Extrakte (aus den Stämmen B13, B18 und B30) milderten die CBPV-Infektion und verbesserten die Überlebensrate der Bienen, während andere Extrakte keine Wirkung hatten (B11 und B49) oder schädlich waren (B69 und B195). In den Säugetierzelllinien hemmte der Extrakt B18 die Replikation von FCV und FCoV in Säugetierzellen und verringerte die Infektiosität von FCoV um ~99%. Der Extrakt B195 verringerte ebenfalls die FCoV-Infektiosität (um ~90%), verursachte aber bei höheren Konzentrationen Zytotoxizität, was die negative Wirkung auf die Überlebensrate der Honigbienen erklären könnte. Die schützende Wirkung der Extrakte B18 und B195 (bei nicht-toxischen Konzentrationen) wurde auch gegen das Influenza-Virus A beobachtet. Im antibakteriellen Test hemmten die Extrakte B18 und B195 das Wachstum von *P. alvei* bei einer Konzentration von 0,39 mg/mL. Die bioaktivitätsgeleitete Dereplikation ergab, dass die Aktivität mit dem Vorhandensein von Diketopiperazinen, einem Siderophor und drei unbekannten Verbindungen korrelierte.

Die Ergebnisse deuten darauf hin, dass die durch Fermentation aus *T. purpureogenus*-Extrakten gewonnenen Verbindungen als prophylaktische oder therapeutische Futtermittelzusätze geeignet sind, um die Resistenz von Honigbienen gegenüber viralen und bakteriellen Krankheitserregern zu fördern. Darüber hinaus schlage ich vor, dass nicht-

pathogene Pilze wie *Talaromyces* spp. und ihre Metaboliten in Bienenbrot eine unerforschte Quelle von Verbindungen darstellen, die eine wichtige Voraussetzung für die Krankheitsvorbeugung sein könnten. Landwirtschaftliche Praktiken, die den Einsatz von Fungiziden beinhalten, können die Pilzgemeinschaft stören und sich daher negativ auf die Gesundheit von Bienenvölkern auswirken.

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I Introduction

Honey bee biology and classification

The origin of bees (Hymenoptera: Apoidea: Anthophila) is dated to the Cretaceous period (about 120 million years ago) and is associated with the transition from a carnivorous diet of their closest relatives, the sphecoid wasps (Hymenoptera: Apoidea: Spheciformes), to a plant-based diet. More than twenty thousand bee species have been described and classified into seven families. The family *Apidae* (Fig. 1, left) involves over six thousand species of honey bees, bumble bees, solitary bees, and stingless bees (Ascher & Pickering, 2022; Cane, 2008). The family is further divided into three subfamilies: *Nomadinae* (cuckoo bees), *Xylocopinae* (carpenter bees), and *Apinae* (Ascher & Pickering, 2022). Within the subfamily *Apinae*, four tribes of bees collect pollen to corbicula (pollen basket): *Euglossini* (orchid bees), *Bombini* (bumble bees), *Meliponini* (stingless bees) and *Apini* (honey bees) (Cane, 2008). Currently, there are eleven accepted species of honey bees, all belonging to the genus *Apis*. Members of this genus are eusocial and some of them are cavity-nesting (Cane, 2008). This characteristic allowed the transition from bee hunting, which is still common in many parts of the world, to beekeeping. The species *A. mellifera* (Linnaeus, 1758) and *A. cerana* (Fabricius, 1793) have become the most commonly kept honey bees.

KINGDOM:	Animalia
PHYLUM:	Arthropoda
CLASS:	Insecta
ORDER:	Hymenoptera
FAMILY:	Apidae
GENUS:	*Apis*

Figure 1: Classification of honey bees of the genus *Apis* (left) and the globally distributed western honey bee *A. mellifera* (right)

The western honey bee *A. mellifera* (Fig. 1, right) is native to Europe, Africa and the Middle East. In these habitats, the bees diverged into more than 25 morphologically and genetically distinguishable subspecies (De La Rua et al., 2006; Garnery et al., 1993). Some of them were introduced to North America between the 1600s and 1922. The commercial North American honey bee populations were formed mainly by three of the subspecies, *A. m. liguistica*, *A. m. carnica* and *A. m. caucasica* (Schiff et al., 1994).

Colonies of *A. mellifera* usually consist of several thousands of workers, one queen, and hundreds of drones. The life cycle of the bees begins when a queen lays a fertilized (female) or unfertilized (male) egg into the wax cell of the honeycomb. The caste differentiation of workers and queens is further determined by the quality and quantity of the food provided to the larvae. The holometabolous development includes five larval instars, followed by cell capping, prepupae, and pupae. The adult emergence occurs approximately after twenty-one (workers), twenty-four (drones), and sixteen (queen) days (Human et al., 2013).

As in many eusocial insects, the distinct castes of a bee colony serve different functions to the superorganism. The function of the queen is mainly to lay eggs after mating with the drones and to produce pheromones to regulate the unity of the colony. The workers perform various tasks in the colony; as young bees, they are engaged with activities in the hive, such as cleaning cells and nursing brood, and building the honeycomb, whereas older bees participate in hive guarding and foraging (Johnson, 2010).

Bee foragers collect nectar, pollen, and resins from a wide range of plants and transform them into products with various health benefits. Six main products are of economic interest: honey, bee pollen, propolis, wax, royal jelly, and venom. Based on their different bioactivities and properties, such bee-derived products find a variety of applications in the bioeconomy. These include the food industry, cosmetics, and biomedicine (El-Seedi et al., 2022). Furthermore, honey bees are important pollinators of agricultural crops and wild plants (Klatt et al., 2013; Klein et al., 2007), far exceeding the value of direct bee products.

The importance of pollination

Pollination is a wind-, water- or animal-mediated transfer of pollen from the anthers (male reproductive organs) to the stigma (female reproductive organs) of flowers of the same species, essential for fertilization and seed production. It is estimated that almost 90% of all flowering plants and at least 35% of agricultural plants are pollinated by animals (Klein et al., 2007; Ollerton et al., 2011). Although many animals are known to pollinate, insects are the most important pollinators on Earth. Nevertheless, a number of reports show alerting declines in insect mass and biodiversity over the past decades (rev. in Wagner et al., 2021). According to the estimations, 75% of the flying insect biomass has been lost over the last three decades (Hallmann et al., 2017) with approximately 40% of all insect species at risk of extinction. These findings raise concern about the consequences of insufficient pollination service in natural and agricultural ecosystems (Potts et al., 2010).

As generalist and prolific pollinators, honey bees visit a wide range of plants and emerged as an effective tool to augment wild pollinators, ensuring stable crop yields and quality for the

agricultural sector (Garibaldi et al., 2009; Klatt et al., 2013; Klein et al., 2007). The number of honey bee colonies has globally increased from 49.2 million in 1961 to the current 101.6 million bee hives (FAO, n.d.). Over the last thirty years, the most significant growth has been observed in Asia (95.7%) and Oceania (89.8%) (FAO, n.d.). Despite the global growth in the number of these human-assisted species, the pollination capacity of honey bees has been outpaced by the rapid growth of the human population (Phiri et al., 2022) and agricultural production that requires pollination (Fig. 2) (Aizen et al., 2008; Aizen & Harder, 2009). Continuance of this trend could be a threat to global food production and security.

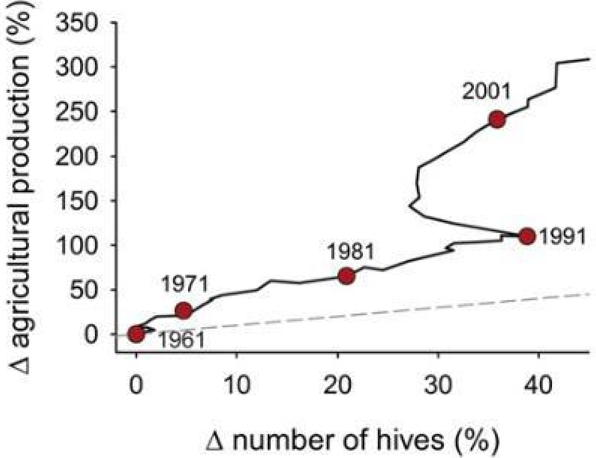

Figure 2: The growth of agricultural production depending on pollinators (y-axis) outpaced the growth of the global stock of managed honey bees (x-axis), as indicated by the increased trajectory above the dashed line, which represents the equal growth rates (Aizen & Harder, 2009)

In addition, significant deterioration in colony health and overall fitness, which is reflected in the poor overwintering success of the bee colonies, reported by beekeepers, particularly in the Northern Hemisphere (Brodschneider et al., 2016; Jacques et al., 2017; Kulhanek et al., 2017; Lee et al., 2015; Steinhauer et al., 2014). Many factors contribute to the losses including exposure to pesticides and their residues, poor nutrition, and parasites and pathogens (Potts et al., 2010; Steinhauer et al., 2018).

Known and emerging pathogens

Honey bees are susceptible to many pathogens, including bacteria, fungi, protozoa, and viruses (Fünfhaus et al., 2018; Quintana, 2015; Ravoet et al., 2013; Steinhauer et al., 2018). The introduction of the ectoparasitic mite *Varroa destructor* (previously called *V. jacobsoni*) from its original host, the Asian honey bee (*A. cerana)* to *A. mellifera* has had devastating consequences

on the colonies of *A. mellifera* in all the continents despite Australia and a few isolated islands (Noël et al., 2020). The mite weakens the bees by feeding on the hemolymph and fat body of developing brood and adults (Ramsey et al., 2019). Furthermore, it releases toxic compounds through saliva and transmits viruses (Forgách et al., 2008; Neumann et al., 2012; Wilfert et al., 2016). Viruses, previously considered a minor threat, have become a serious problem for beekeepers since the introduction of the *Varroa* mites (Budge et al., 2020; Genersch & Aubert, 2010; Noël et al., 2020; Teixeira et al., 2008).

Currently, over twenty-four viruses have been described in bees. Most of them are positive-sense single-stranded RNA viruses from the order Picornavirales, family *Dicistroviridae*, and *Iflaviridae*, such as deformed wing virus (DWV), Israel acute paralysis virus (IAPV), acute bee paralysis viruses (ABPV) sacbrood virus (SBV) (Fig. 3). Some viruses are unclassified, e. g. chronic bee paralysis virus (CBPV), Lake Sinai virus (LSV), and *Apis mellifera* filamentous virus (AmFV). Novel viruses from other families have been found in recent studies (Galbraith et al., 2018; Mordecai et al., 2016; Ravoet et al., 2013; Remnant et al., 2017), such as *Apis mellifera* flavivirus (AFV), rhabdovirus (ARV) and bunya virus (ABV)(Remnant et al., 2017).

The bacterial diseases American foulbrood (AFB) and European foulbrood (EFB) caused primarily by *Paenibacillus larvae* and *Mellisococcus plutonius*, respectively, are highly contagious and affect the brood of honey bees. The outbreak of bacterial diseases, in particular AFB, can lead to significant economic losses and must be announced to the veterinary or competent authority ("World Organisation for Animal Health - Diseases, Infections and Infestations Listed by WOAH," 2023).

Fungal pathogens of honey bees include *Nosema ceranae*, affecting the bee digestive system, *Ascosphaera apis* causing chalkbrood disease and *Aspergillus* spp. associated with stonebrood disease (Quintana, 2015). Bee pathogenic protozoa include trypanosomatids *Lotmaria passim* and *Crithidia mellificae*, neogregarine *Apicystis bombi*, and the amoeba *Malighamoeba mellificae* (Ravoet et al., 2013; Schwarz et al., 2015).

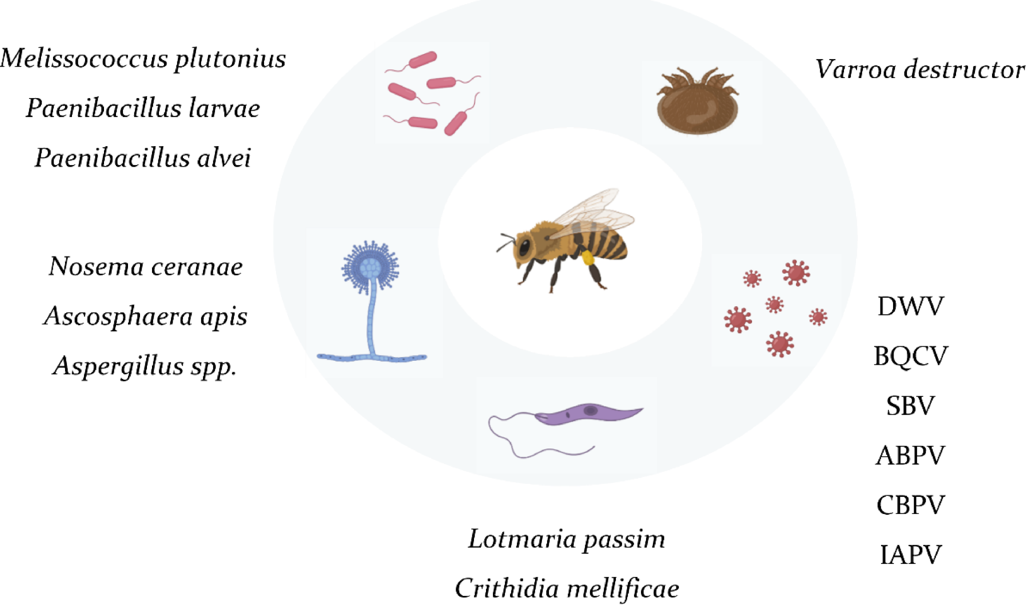

Figure 3: Honey bee colony health is threatened by parasites, viruses, bacterial, fungal, and protozoan pathogens. The figure shows the most studied representatives of each group. Created with BioRender.com

The global change and anthropogenic activity facilitate the transmission of non-native species that can pose a threat to *A. mellifera* colonies. In 2004, the Asian hornet *Vespa velutina* (Hymenoptera: Vespidae; Lepeletier, 1836) was accidentally introduced to France with goods from China. Since then, it has spread to other European countries (Keeling et al., 2017). This is problematic because *V. velutina* is both, a predator of honeybees, and it carries novel pathogens able to infect bees (Marzoli et al., 2021). Several monitoring programs aim to mitigate its rapid range expansion to minimize losses among honeybee populations (Cappa et al., 2021; Dalmon et al., 2019).

Attention has been also drawn to the small hive beetle *Aethina tumida* (Coleoptera: Nitidulidae; Murray, 1867). The damage is particularly caused by the larvae that develop in the hive and feed on food stores and brood while damaging the combs and spoiling the honey stores, which can lead to colony abduction (World Organisation for Animal Health (OIE), 2018). Moreover, the beetles can transmit viruses (Eyer et al., 2009). In its native range, South and Central Africa, the beetle poses only a minor threat for the local bee colonies (Neumann & Elzen, 2004). However, considerable damages have been reported in its non-native range in the U.S. and Australia (Neumann et al., 2010). Furthermore, in 2014, *A. tumida* was introduced to the EU (*Istituto Zooprofilattico Sperimentale Delle Venezie (IZSVe)*, n.d.) and researchers expect climate change to facilitate the spread to regions of North Africa and Southern Europe (Jamal et al., 2021).

The antipathogenic defense systems of honey bees

Innate immunity

Potential pathogens must overcome several morphological barriers, such as the outer, hair-covered chitin cuticle and the inner, hostile gut environment and epithelium. Beyond these barriers, innate immunity in honey bees includes humoral and cellular immune responses (Imler et al., 2006). Cellular immunity involves processes executed by different types of hemocytes: (a) granular cells are primarily involved in phagocytosis, (b) plasmatocytes are responsible for phagocytosis, wound healing, and melanization, and (c) oenocytoids are mainly involved in melanization. The humoral response involves clotting and melanin synthesis. Furthermore, innate immunity is associated with the production of different antimicrobial peptides (AMPs)(Kojour et al., 2020).

AMPs are usually small peptides (>10 kDa) synthesized by a major immune-responsive organ – the fat body. The synthesis is followed by secretion to the hemolymph where the AMPs reach their effective concentration (Lemaitre & Hoffmann, 2007). In *Drosophila*, about twenty AMPs have been identified and grouped into seven classes – diptericins, attacins, drisocins, cecropins, defensins, drosomycins, and metchnikowin. In honey bees, four classes of AMPs have been described to date – apidaecins, abaecin, defensins, and hymenoptaecin. Interestingly, many more genes encoding AMPs have been found in the genome of *A. cerana* (87 genes) in these families compared to *A. mellifera* (16 genes). The gene repertoire is known to encode around 25 AMPs in *A. cerana* and 11 AMPs in *A. mellifera*. The main difference is in hymenoptaecin family; 13 peptides are generated in *A. cerana* and only 1 in *A. mellifera* (Xu et al., 2009). Several AMPs are also components of bee venom, such as melittin (40–60% of the venom dry weight), apamin, adolapin, and secapin (Danihlík et al., 2015; Gauldie et al., 1976).

Several immune signaling pathways have been identified in honey bees: Toll, Immune deficiency (IMD), Jun N-terminal kinase (JNK), Janus kinase-signal transducer (JAK-STAT), and RNA interference (RNAi). In most cases, the pathways are activated by pattern recognition receptors (PRRs). These host PRRs recognize the pathogen-associated molecular patterns (PAMPs), conserved molecular structures that are usually part of the cell walls of the potential pathogens, e.g., lipopolysaccharides (gram-negative bacteria), lipoteichoic acid (gram-positive bacteria), dsRNA (viruses) and ß-glucans (fungi) (Kojour et al., 2020).

The analysis of the genome (The Honeybee Genome Consortium, 2006) revealed a significantly reduced number and diversity in immune gene families in *Apis mellifera* (71 genes) compared to other insect species, such as *Drosophila melanogaster* (209 genes) and *Anopheles gambiae* (196 genes) in each stage of immunity reaction. To date, around 200 immune genes

have been described in the genomes of *Apis* spp. (144 – 209) (Diao et al., 2018; Henriques et al., 2021) and *Bombus* spp. (184 – 194) and the counts are much lower compared to over 350 genes described in *D. melanogaster* (364) and *A. gambiae* (378) (Sun et al., 2020). The reduced immune repertoire may reflect, among other things, the ability of social defense mechanisms in honey bees to reduce pathogen pressure and thus the dependence on individual immunity in honey bees (Claudianos et al., 2006). Nevertheless, a comparison of immune repertoire in different bee and bumble bee species revealed striking similarities, regardless the level of sociality (Barribeau et al., 2015).

Social immunity of the colony

Social immunity involves behavioral defense mechanisms of the individuals, such as the release of alarm pheromones to alert other colony members in case of danger, guarding, swarming, grooming, and hygienic behavior. The hygienic behavior, i.e., detection, uncapping, and removal of the infected pupae, is considered a primary resistance mechanism to control diseases, such as American foulbrood and chalkbrood disease as well as the *Varroa* infestation. During grooming, bees actively remove the phoretic mites from their own body (autogrooming) or body of other bees (allogrooming) (Oldroyd, 1996; Spivak, 1996). While these traits are of interest in the selection breeding programs, some defense mechanisms, such as swarming are rather undesirable.

Honey bees also promote colony resilience by utilizing bioactive compounds in the hive environment and food stores. They collect nectar, pollen, and propolis from a variety of plants, resulting in the presence of a wide range of phytochemicals with antimicrobial properties. Furthermore, bees increase the antimicrobial potential of bee products by adding antimicrobial enzymes such as glucose oxidase (GOX), which further catalyze the production of hydrogen peroxide (H2O2) (rev. in Gaubert et al., 2023).

The role of therapeutics in the control of bee pathogens

Responsible beekeeping requires knowledge of best practices to ensure the health and productivity of bees, and the safety of bee products for human consumption. These practices are applied in general apiary management, sanitation, feeding and watering, record keeping, and disease and pest management (FAO and IZSLT, 2021).

The use of acaricides

The spread of *V. destructor* to *A. mellifera* colonies has become a problem of pivotal importance, requiring a rapid solution. Therefore, chemical treatments, such as amitraz and fluvalinate, have been implemented as first aid in apiculture, despite concerns about the possible risk of resistance development and contamination of hive products (Boecking & Genersch, 2008). Commercially available synthetic acaricides include formamidine amitraz (Apivar), the organophosphates coumaphos (CheckMite+), pyrethroids tau-fluvalinate (Apistan), acrinathrin, or flumenthrin (Bayvarol). All these compounds are lipophilic, meaning they can be absorbed by beewax, but have a very low risk of residue accumulation in the watery honey (FAO and IZSLT, 2021; Haber et al., 2019; Williams, 2000).

As an alternative to synthetic acaricides, several naturally occurring compounds are used for *Varroa* control. These include organic acids, i.e., lactic and formic acid, oxalic acid, and the essential oil thymol or hop oil (FAO and IZSLT, 2021; Haber et al., 2019; Williams, 2000). Because these compounds are hydrophilic or volatile, they are unlikely to accumulate in comb wax and cause resistance development. However, their application and use as acaricides within the beehive is complex (Rosenkranz et al., 2010).

Besides the above-outlined molecular approaches to mite control, various non-chemical practices are used to reduce the *Varroa* infestation. These methods include removal of drone brood, splitting colonies, or using a screened bottom board. However, the use of acaricides remains the most effective method and therefore remains an important part of beekeeping management (Haber et al., 2019).

Antibiotics and other anti-infectives

The therapy against AFB is controversial; some authorities consider the destruction of infected colonies to be the only effective control measure, while others suggest therapeutic option such as biotechnological methods or even the therapeutic or prophylactic use of antibiotics. However, the use of antibiotics cannot ensure the complete disinfection of the colony because they have rather bacteriostatic effect and are not effective against spores (AFB) or capsules (EFB). Moreover, improper dosing increases the risk of antimicrobial resistance and the presence of residues in hive products (Williams, 2000).

Nowadays, three antibiotics are approved by the U.S. FDA (Food and Drug Administration)(FDA, 2023): oxytetracycline (approved 1996), tylosin (approved 2005) and lincomycin (approved in 2012). Nevertheless, the use of antibiotics is banned or strictly regulated in many countries (Reybroeck et al., 2012).

Another antimicrobial drug, fumagillin (Fumidil B), can be used to suppress the *Nosema*

infection (Williams, 2000).

Alternative ways to control bee pathogens

The non-chemical control methods include particularly selective breeding of varroa-tolerant colonies, dsRNA to trigger RNAi, the use of probiotics, and the use of natural products (FAO and IZSLT, 2021). Natural products are screened for biologically active compounds that can augment the repertoire of disease management in human medicine as well as in livestock (Eichberg et al., 2022). Honey bees as generalists collect nectar and pollen from a wide range of plants and thus come across various biologically active phytochemicals. Their presence is important for bee immunity, detoxification, and influences the antimicrobial and antioxidative properties of bee products. In addition, honey bees are in contact with compounds produced by the gut microbiome as well as the microbes from the environment, colonizers of the hive surfaces, and food stores. A handful of studies have focused on the function of the microorganisms that are present in the hive, in particular the filamentous fungi. In fact, several fungal bee cohabitants, such as *Penicillium*, *Aspergillus* and *Cladosporium* (Frisvad et al., 2004; Mandal et al., 2022; Salvatore et al., 2021) are well-known producers of various antimicrobial compounds that might have an impact on the microbial balance (Paludo et al., 2019) in the hive and the bee resistance against chalkbrood disease (Disayathanoowat et al., 2020; Gilliam et al., 1988). A better understanding of the bee-associated fungal community and their compounds could benefit bee health research as well as natural product discovery.

The aim and objectives of the study

The aim of this work was to characterize strains of *Talaromyces purpureogenus* isolated from *A. mellifera* bee bread in the Czech Republic and to evaluate their antimicrobial potential against bee pathogens. The genus *Talaromyces* is ubiquitous and colonizes mainly soil, and food but also marine water (Zhai et al., 2016). Despite its ubiquity, there is only few reports on *Talaromyces* spp. presence in *Apis* spp.; they were found in the honey of *A. mellifera* (Rodríguez-Andrade et al., 2019), bee bread of *A. cerana* (Disayathanoowat et al., 2020), and dead adults of *A. dorsata* (Fabricius, 1793) (Sandeepani & Ratnaweera, 2020). Here, I describe the first detection of *T. purpureogenus* in *A. mellifera*, specifically in bee bread.

It is noteworthy that *Talaromyces* spp. are producers of a wide array of biologically interesting compounds. Indeed, the genus has been reported to produce over two hundred compounds and many of them have antioxidant and antimicrobial activity (Lan & Wu, 2020; Zhai et al., 2016). This fact motivated my research towards the exploration of biological activities

of the isolated strains against bee pathogens of bacterial, fungal, and viral origin, whose control is problematic or not available. To be able to evaluate the activity of the compounds, I extracted the *T. purpureogenus* strains with methanol and used the extracts in the experimental assays in multiple ways. First, I tested the effect of extracts' dietary addition on laboratory-kept CBPV-infected bees. Subsequently, the results were verified in an independent system, using mammalian cell lines and viruses. To test the antibacterial and antifungal activities, I implemented the standard *in vitro* methods, the micro-broth dilution method against different strains of *Paenibacillus* spp., and the disk diffusion method against three *Aspergillus* spp. The antibacterial activity test was followed by the evaluation of the specific active compounds using the bioactivity-guided dereplication.

The results of the studies are summarized in the following section (chapters 2 and 3). Furthermore, I included results of publications, which I contributed to and which are related to pathogen prevalence and virus pathogenicity evaluation in honey bees, and therefore fit well to the whole context of this work.

II Summary of the publications

Each chapter of the following section summarizes the results of research articles, which were already published by established peer-reviewed journals. In the first part I helped to evaluate the prevalence of pathogens in Czech Republic (Chapter 1). In the main part, I characterize distinct *T. purpureogenus* strains from *A. mellifera* bee bread and explore activities of their extracts against bee pathogenic CBPV (Chapter 2), as well as fungi and bacteria (Chapter 3). In the last part, I was involved in the evaluation of the pathogenicity of Culex Y virus in bees, to verify the safety of its potential application in the biological control of mosquitoes (Chapter 4).

Chapter 1

Screening of honey bee pathogens in the Czech Republic and their prevalence in various habitats

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Petr Mraz, Marian Hybl, Marek Kopecky, Andrea Bohata, Irena Hostickova, Jan Sipos,
Katerina Vocadlova and Vladislav Curn

Insects. 2021 Nov 24;12(12):1051. doi: 10.3390/insects12121051. PMID: 34940139; PMCID: PMC8706798.

Many abiotic and biotic stressors jeopardize health and survival of the bee colonies. Among others, pathogens and the parasite *V. destructor* are one of the main drivers of the colony losses. The aim of the study was to evaluate the prevalence of the pathogens in various habitats, i.e., agricultural land, urban ecosystem and national park in the Czech Republic. The screening was performed in 250 hives (50 apiaries) in the fall 2019. A set of specific primers was used to detect the eukaryotic pathogens (*Nosema* spp., *A. apis*, *C. mellificae* and *L. passim*), bacterial pathogens (*P. larvae* and *M. plutonius*) and viruses (DWV, BQCV, CBPV, ABPV, SBV, LSV and MLV - Macula-like virus).

The eukaryotic pathogens' burden was the highest in urban area, followed by agricultural land and lowest in national park. Surprisingly, the eukaryotic pathogens, i.e., *L. passim* (72%), *N. ceranae* (64%) and *C. mellificae* (38%), were predominating pathogens in all the types of habitats. Conversely, the bacterial pathogens, *M. plutonius* and *P. larvae* were detected at the lowest prevalence only in the urban area and agricultural land (only *M. plutonius* detected). In the national park, the causative agents of the foulbroods were not detected. Furthermore, no clinical symptoms of the bacterial brood diseases were observed in any of the tested apiary.

While the prevalence of the bacterial and eukaryotic pathogens differed in each habitat, the prevalence of viruses was comparable in all three habitats. The most prevalent viruses were DWV-A and ABPV, followed by DWV-B, LSV and MLV. The DWV is considered one of the most harmful viruses that actively replicates and transmit through the *Varroa* mites and causing wing deformities. The most prevalent variant to be the DWV-A were found in all the types of habitats. Nevertheless, the pCCA analysis revealed the association between the colony mortality and two other less prevalent variants, DWV-C and DWV-B. The average winter mortality in the urban area and agroecosystem (24.51% and 21.50%, respectively) was twice as high as in the national park (11.11%).

Chapter 2

Extracts of *Talaromyces purpureogenus* strains from *Apis mellifera* bee bread inhibit the growth of *Paenibacillus* spp. *in vitro*

Katerina Vocadlova, Tim Lüddecke, Maria A. Patras, Michael Marner, Christoph Hartwig, Karel Benes, Vladimir Matha, Petr Mraz, Till F. Schäberle, Andreas Vilcinskas

Microorganisms. 2023 Aug 11;11(8):2067. doi: 10.3390/microorganisms11082067. PMID: 37630627; PMCID: PMC10459140.

Fungi in bee hives have an important function in nutrition as they participate in pollen fermentation and its transformation to the more easily digestible bee bread. Furthermore, some genera are well-known producers of bioactive compounds that can affect honey bee health and resistance to pathogens.

In this study, I characterized seven strains of *T. purpureogenus* from the bee bread from *A. mellifera* and explored their antimicrobial potential. Specifically, I tested the activity of methanol extracts of the strains against fungi *Aspergillus* spp. and the bee-pathogenic bacterium *P. alvei*, using the standard disk diffusion (antifungal activity) and microbroth dilution (antibacterial activity) methods. I observed no effect on the fungal pathogens. Nevertheless, the extracts from the *T. purpureogenus* strains B18 and B195 inhibited the growth of *P. alvei* at a minimum inhibitory concentration (MIC) of 0.39 mg/mL. To support the results of the antibacterial activity, the screening panel was augmented by including further *Paenibacillus* species. Interestingly, extract B18 inhibited the growth of *P. lautus* and *P. lactis* at MIC = 0.5 mg/mL. The activity of the extract B195 was also observed, yet only at the highest tested concentration (MIC = 2 mg/mL). To investigate the bioactive compounds, subsequent micro-fractionation, and

bioactivity-guided dereplication were performed. The molecular formulae corresponding to the major ions in the active fractions were identified as diketopiperazines, 5,6,8-trihydroxy-3-methyl-2-benzopyran-1-one, a siderophore, and three unknown compounds. Furthermore, two compounds retrieved more than 100 candidates in the database and could not be assigned.

In a course of the study (2019-2020), further bee bread sampling was performed in spring and summer in areas with various anthropogenic pressure (rural area, city periphery, urban and suburban area) to briefly assess the seasonal presence of *Talaromyces* spp. by cultivation methods. Strains of *T. purpureogenus* were found in the rural area, city periphery, and the urban area. Two *Talaromyces* species were isolated from the urban area – *T. purpureogenus* and *T. piceae*. Interestingly, all the *Talaromyces* spp. were found only in spring.

Chapter 3

Crude extracts of *Talaromyces* strains (Ascomycota) affect honey bee (*Apis mellifera*) resistance to chronic bee paralysis virus

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Katerina Vocadlova, Benjamin Lamp, Karel Benes, Vladimir Matha, Kwang-Zin Lee, and Andreas Vilcinskas

Viruses. 2023 Jan 25;15(2):343. doi: 10.3390/v15020343. PMID: 36851556; PMCID: PMC9958978.

Viruses have become a major problem with the expansion of *V. destructor* that facilitate their reproduction and transmission. As a result, viruses are one of the main factors affecting bee health and overwintering success. The detection of viruses is not easy due to the common lack of clinical symptoms in the hive. Furthermore, no therapeutics are available to treat the viral infection and the current control is part of the management of *V. destructor* infestation. Despite the successful control measures against *V. destructor*, such as using "hard" and "soft" chemical treatments or non-chemical biotechnological measures, mite infestation and the associated viral infections are an annually recurring problem in beekeeping.

In this research article, I have evaluated the antiviral activity of crude extracts of seven *T. purpureogenus* strains against CBPV. The experiment was performed with caged workers of *A. mellifera*. The newly-emerged bees were fed the extract-enriched diet, followed by injection with CBPV, and daily monitored survival. Crude extracts from three *T. purpureogenus* strains (B13, B18, and B30) mitigated the viral infection and increased the survival rate of the bees, whereas other extracts had no effect (strains B11 and B49) or were toxic (strains B69 and B195). The effects of the extracts were further studied *in vitro* against feline calicivirus (FCV) and feline coronavirus

(FCoV) in Crandell-Rees feline kidney cells (CRFK). The addition of the extract from strain B18 inhibited the replication of both viruses. Furthermore, extracts from the strains B18 and B195 reduced the infectivity of FCoV by ~99% and ~90%, respectively.

Antiviral activity of the extracts against influenza viruses (unpublished data)

To support the results of the antiviral activity assay, the extracts were tested against influenza viruses in Madin-Darby canine kidney cells subclone II (MDCK II) by the research group of Dr. Kornelia Hardes (Fraunhofer IME, Gießen). Briefly, the cells were cultivated in bench stable Glutamax supplemented Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and streptomycin and kept at 37 °C under 5 % CO_2 atmosphere. Cells were split at least twice a week at a ratio of 1:10. The cells were inoculated with influenza viruses A (H3N2 and H1N1) and B (B/Mal and B/Mass) for 1 h followed by the treatment with the samples for 48 h as described above at 37 °C and 5% CO_2 in infection media (DMEM GlutaMAX supplemented with 100 U/ml penicillin/streptomycin and 0.2% Bovine Serum Albumin (BSA, Carl Roth). Cell viability was determined by quantification of the ATP content using the CellTiter-Glo Luminescent Cell Viability assay (Promega) according to the manufacturer's instructions. Values of virus-treated cells were set as a baseline and the data were normalized to aprotinin set to 1 (n = 3). At the tested concentrations, the extracts B18 and B195 had a protective effect against influenza A viruses (Fig. 4).

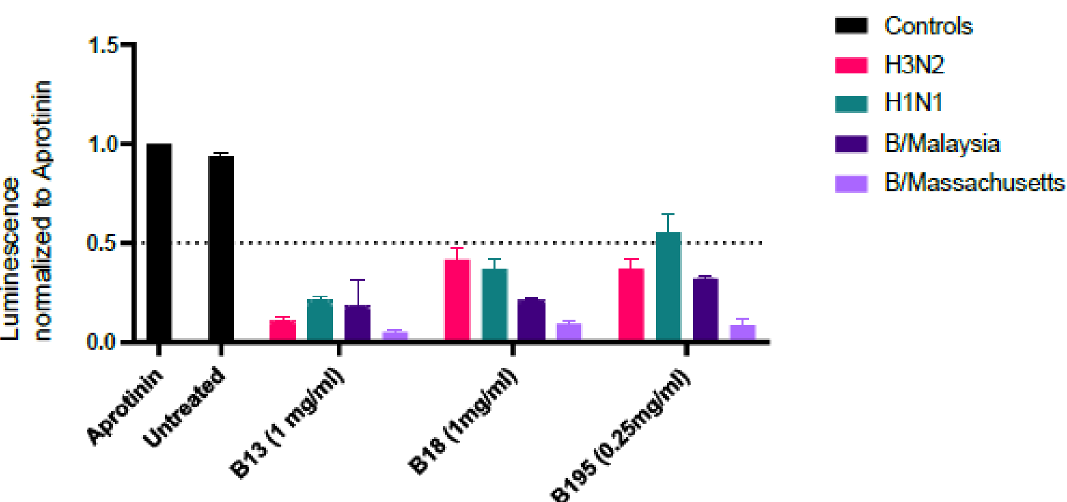

Figure 4: The extracts from *T. purpureogenus* strains B18 and B195 showed protective effects against influenza A viruses (H3N2 and H1N1) in MDCK II cells. (Created by MSc. Johanna Eichberg).

Chapter 4

Culex Y virus: A native virus of *Culex* species characterized *in vivo*

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Viruses. 2023 Jan 14;15(1):235. doi: 10.3390/v15010235. PMID: 36680275; PMCID: PMC9863036.

Mosquito-borne diseases pose serious problems to human and livestock populations in many parts of the world and their incidence has been increasing with the expanding geographical range of mosquitoes. The use of insect-specific viruses as a biological control of insect vector populations has received increased attention in the past years as an alternative to chemical insecticides. This study focused on the potential candidate, Culex Y virus (CYV), and its *in vivo* characterization in mosquitoes *Cx. pipiens*. To evaluate the specificity of the virus, additional *in vitro* and *in vivo* tests were performed. My part consisted of the analysis of the survival rate of the CYV-infected *A. mellifera*. The newly-emerged bees were briefly anesthetized with CO_2 and injected (Nanoject II, Drummond) into their thorax with 1 µL of the viral suspension or Schneider's Drosophila Medium as a negative control. The bees were transferred into the plastic boxes (11.5 × 8.5 cm × 8 cm) with a piece of wax, and provided with sucrose solution (1:1) *ad libitum*. The mortality was recorded every day for 14 days. During the experiment, six living bees were collected at four time points – 0, 3, 7, and 14 dpi (days post injection) and sent on dry ice to the Institute for Parasitology, University of Veterinary Medicine Hannover, Germany for the qRT-PCR. No difference was observed between the mortality of the virus-infected bees and the control (Log-rank: $p=.7166$). In line with this finding, the injection with CYV (~ 8.17×10^5 RNA copies) did not lead to an increase in the viral RNA copies and decreased by an order at the end of the experiment (~ 8.86×10^4 on 14 dpi).

III Discussion

Honey bee biology has been studied for centuries and continues to be of high interest due to the benefits that bees and their products provide. With the analysis of the bee genome in 2006 (The Honeybee Genome Consortium, 2006) and the development of molecular biology techniques, considerable advances in our understanding have been made. These have benefited many aspects of honeybee research in the last decades, such as physiology and immunity, bee behavior, bee-associated symbiotic and pathogenic microorganisms, or the impact of pesticides exposure (Dickey et al., 2023; Romero et al., 2019).

Interestingly, the most prevalent pathogens in the Czech Republic were the trypanosomatids *L. passim* and *C. mellificae*. Despite the lack of clear knowledge about the harm these eukaryotes cause to the bees (Michalczyk et al., 2020; Schwarz et al., 2015), studies demonstrated their contribution to colony losses (Gómez-Moracho, T. Buendía-Abad et al., 2020) and disruption of the composition of symbiotic gut microorganisms (Hubert et al., 2017). The co-infection of trypanosomatids and *N. ceranae*, which was also highly prevalent in the apiaries has been shown to reduce the immune gene expression (Arismendi et al., 2016; Ravoet et al., 2013; Vejnovic et al., 2018). Other fungal strains, *N. apis* and *A. apis* were not detected. The eukaryotic bee pathogens were found mainly in the urban area (town) and the lowest prevalence as well as species richness was observed in the national parks. In the case of viruses, the prevalence was similar in all the tested habitats. The most prevalent viruses were DWV (in particular variant DWV-A), ABPV, and LSV, followed by SBV, CBPV, and BQCV. The data revealed an association between the presence of less prevalent variants of DWV, DWV-B, and DWV-C, and the colony mortality.

Many viruses have been detected in bees, including the plant virus tobacco ringspot virus (TRSV) (Galbraith et al., 2018; Li et al., 2014), although the adaptation of this virus to bees as hosts is not well supported by genetic data (Cornman, 2017). Host specificity and pathogenicity studies are an important precaution for biological control methods (Brodeur, 2012). The use of viruses as biological control agents against pests and disease vectors has become a promising approach as an alternative to chemical insecticides (Abd-Alla et al., 2020). However, to avoid the risk of infection of non-target insects, such as bees, the host specificity of the virus should be carefully evaluated (Brodeur, 2012). To the best of my knowledge, the CYV has never been detected in honey bees. The results from chapter 4 show no effect of this virus on the survival rate of *A. mellifera* as well as no viral replication within this host. Thus, the data represent the first step of the safety assessment of the CYV application for mosquito control. Nevertheless, more in-depth testing involving would be required prior to the field application.

Besides the number of studies on bee pathogens, the last decades have brought a plethora of findings about bee-associated microorganisms with undoubted beneficial role in honey bees. Indeed, gut microorganisms not only contribute to nutrient utilization, but also influence the immunity and resistance of the bees to diseases, and shape the behavioral tasks of bees (Jones et al., 2018).

Yet, honey bees are surrounded by many other microorganisms found on their bodies, on hive surfaces, and in food stores, whose function is rather unknown (Smutin et al., 2022). Not much attention has been paid to the filamentous fungi among them. In the previous section, I summarized the results of the work presenting the first isolation and characterization of *T. purpureogenus* strains from the bee bread of *A. mellifera* and the antimicrobial activity of their extracts.

The genus *Talaromyces* and its association with honey bees and other insects

The species *T. purpureogenus* (Eurotiales: Trichomaceae; fig. 5; Samson, Yilmaz, Frisvad and Seifert 2011) from the genus *Talaromyces* was formerly known as *Penicillium purpureogenum* (Stoll, 1904). It belongs to the section *Talaromyces*, one of the seven sections of this genus (Yilmaz et al., 2014).

KINGDOM:	Fungi
PHYLUM:	Ascomycota
CLASS:	Eurotiomycetes
ORDER:	Eurotiales
FAMILY:	Trichocomaceae
GENUS:	*Talaromyces*

Figure 5: Classification of the genus *Talaromyces* (left) and the microscopic picture of the biverticillate conidiophores of *T. purpureogenus* dyed with cotton blue (right).

Herein, I describe the presence of *T. purpureogenus* in *A. mellifera* for the first time. Although no evidence of the genus *Talaromyces* in *A. mellifera* bee bread had been reported before my investigations, its presence is not surprising because bees can collect *Talaromyces* spp. from many sources; foragers passively or actively collect fungi and incorporate them into corbicular pollen (Parish et al., 2020; Shaw, 1990). Interestingly, some fungi are likely introduced or eliminated by bees during pollen collection and storage (M. Gilliam et al., 1989). I have identified two *Talaromyces* species – *T. piceae* and *T. purpureogenus* using our cultivation-based

method. Nevertheless, the relatively low number of positive bee bread samples (the highest prevalence was ~15% in April 2020) rather contradicts the assumption that these fungi are part of the core fungal community in the bee bread. Seasonal factors might play a role, as the *Talaromyces* strains were detected only in spring. Other studies of fungal composition in bee bread in temperate climates did not detect any *Talaromyces* spp. However, they were conducted in summer, where the absence of *Talaromyces* is consistent with our results. In worker bees, the prevalence of fungi was higher in fall and winter (Martha Gilliam, 1997), however, no study has explored the composition and abundance of fungi in bee bread during the months of no-flight activity.

It is also noteworthy that the negative samples were taken from hives near agricultural land. The presence of agricultural land, such as rapeseed field, increase the risk of fungicide residues in the food stores and may therefore affect the fungal community. The detrimental effect of agricultural pressure and fungicide contamination on the fungal community in bee bread has been demonstrated by different methods (Dimov et al., 2021; Yoder et al., 2013). Nevertheless, more data are needed to conclude the presence and seasonal prevalence of *T. purpureogenus* and other *Talaromyces* species in bee bread.

Antiviral activity of the *T. purpureogenus* strains

In the *in vivo* experiment, investigating the effects of *T. purpureogenus* extracts on CBPV infection, diverse biological effects were observed. The addition of three crude extracts to the diet reduced the mortality rate and extended the lifespan of CBPV-infected bees (B13, B18, and B30); other extracts had no significant effect (B11 and B49) or were toxic to the bees (B69 and B195), resulting in increased mortality. A similar pattern was observed when only the extracts were injected into bees during the protocol optimization phase. Nine or ten newly-emerged bees were injected with 4 µL of the fungal extracts (8 mg/mL) or acetone as a control (8%) into the thorax, and mortality rates were monitored for ten days. Although the sample size was small, the positive effect of extract B18 and the detrimental effect of extract B195 on bee survival could be seen (Fig. 6).

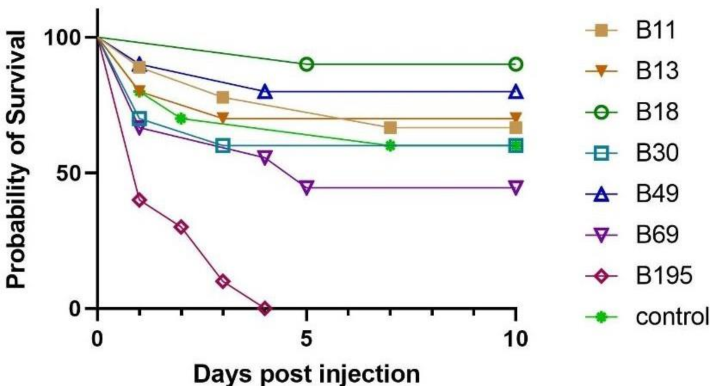

Figure 6: Survival rate of honey bees injected with extracts of *T. purpureogenus* or acetone only (solvent control). The results of this small-scale preliminary test indicated positive as well as negative effect of extracts derived from the strains B18 and B195, respectively.

To evaluate the antiviral activity of the extracts while excluding the combination of possible direct and indirect effects in the *in vivo* experiment, the test was performed in an established cell culture model. In the first assay, the extract-treated and untreated CRFK cells were exposed to the enveloped and non-enveloped viruses, FCV and FCoV, respectively. Subsequently, the destruction of the cell monolayer caused by viral lysis in all the treatments except extract B18 was observed. This extract (from the *T. purpureogenus* B18 strain) repressed viral replication in a dose-dependent manner. The actual virucidal effect of the extracts was observed in the second viral inactivation assay, where extract B18 and B195 inactivated ~99% and ~90% of FCoV viral particles, respectively. The extracts also improved viability of cells infected with influenza A virus H1N1 and H3N2. Further analyses aim to identify the active compounds from the crude extracts. In addition, beneficial effect of some extracts on the survival rate of CBPV-infected that could not be linked to antimicrobial activity and remains to be explored.

Antibacterial and antifungal activity of the *T. purpureogenus* extracts

The crude extracts from the *T. purpureogenus* strains were tested against fungi and bacteria – species of *Aspergillus* and *Paenibacillus*. With the method used, I did not detect the antifungal activity of the extracts. In the antibacterial test, extracts B18 and B195 inhibited the growth of bee pathogen *P. alvei*, as well as *P. lautus* and *P. lactis*. However, the activity of extract B195 was observed only at a higher concentration (2 mg/mL) against the latter two bacteria. Bioactivity-guided dereplication of extract B18 was performed with *P. lautus*, so we assume, albeit with caution, that the compounds are likely active against the other *Paenibacillus* species as well. The compounds identified in the active fractions were diketopiperazines, a siderophore,

and three unknown compounds. Siderophores have attracted considerable attention because of their potential applications in medicine and environmental field (Kurth et al., 2016). These metal chelators are important for the uptake, transport, and storage of iron in plants, bacteria, and fungi. Fungal siderophores are usually acylated hydroxamates (Haas, 2003), such as coprogen-type talarazines produced by *Talaromyces* (Kalansuriya et al., 2017). The biosynthesis of siderophores influences the virulence of fungi and their interaction with other microorganisms (Haas, 2014). The ability to synthesize iron-chelating molecules can cause suppression of growth in some microbes as a result of starvation of this essential nutrient. Conversely, providing the source of iron to other microbes, such as yeasts, can promote their growth (Haas, 2014). Several fungal compounds have been linked to the regulation of the microbiota in bee bread, such as lovastatin (Paludo et al., 2019) and organic acids (Disayathanoowat et al., 2020). Given the inhibitory activity of the siderophore against the bacteria tested in our study, it would be interesting to investigate the use of siderophores to regulate AFB and EFB *in vivo*. Furthermore, the unknown active compounds should also be investigated as novel antimicrobials.

Toxicity of the *T. purpureogenus* strains

In general, the extracts obtained from the used *T. purpureogenus* strains had diverse biological effects in our experiments. Extract B18 had an antimicrobial activity in all assays performed (except the antifungal activity assay), while it had no toxic effect on the bees and mammalian cells. Conversely, a virucidal activity of extract B195 was observed; however, its high cytotoxicity prevented the determination of antiviral properties in the other assays. Subsequent chemical profiling revealed, that most of the *Talaromyces* strains produced rubratoxin A and B (B11, B30, B49, B69, and B195), while the two extracts B13 and B18 contained no rubratoxins or their concentration was below the detection limit of the method used. The latter two extracts had a positive effect on bee survival. It is worth noting that these strains are also strong producers of *Monascus*-like red pigments that are relevant to the food industry. Honey bees are exposed to many types of mycotoxins, such as aflatoxins, ochratoxins, deoxynivalenol, zearalenone, and fumonisins produced by *Aspergillus*, *Penicillium*, and *Fusarium* species (Kostić et al., 2019). Under natural conditions, mycotoxins are diluted in food stores to a concentration that is tolerable by bees and the toxicity can also be mitigated by propolis (Niu et al., 2011). Our results suggest that the presence of rubratoxins may increase the mortality rate of the bees. However, extracts B30 promoted bee survival despite the presence of rubratoxins, so the interactions appear to be more complex. The concentration of rubratoxins in the extracts, as well as the concentration that bees can tolerate, remain unknown and should be investigated in further trials.

Conclusions and future perspectives

This work presents the first description of *Talaromyces* strains in bee bread of *A. mellifera* and provides novel insights into the relationship between fungal bioactivity and bee health under laboratory conditions. The presence of *Talaromyces* strains in bee bread can probably vary depending on the season and location of the apiaries. It is important to note that observations of fungal growth in bee bread have differed; some researchers observed only fungal spores in bee bread (Anderson et al., 2014), while others observed spore germination and production of short hyphae (< 10 µm) (M. Gilliam et al., 1989). Bee bread is an unfavorable substrate for microbial growth due to low water activity (a_w, available water for microbial growth), acidic pH, high oxidation-reduction potential (Anderson et al., 2014). However, some microorganisms, particularly yeasts and molds, are able to grow slowly under such conditions. Some *Talaromyces* species can grow at a water activity of 0.82, while other fungi can grow at a water activity as low as 0.61 (Rodríguez-Andrade et al., 2019). In addition, the nutrients-rich substrate like bee bread, as well as the absence of competing bacteria under these conditions, may extend the range of temperatures and water activities suitable for spore germination and growth (Beuchat, 1983). Interestingly, some bee-associated fungi have greatly reduced fruiting bodies, likely as a result of adaptation on the bee habitat (Wynns, 2015).

By producing compounds with antibacterial and antiviral activity, strains of *T. purpureogenus* could protect bees against some pathogens. Nevertheless, by producing mycotoxins (rubratoxin A and B), the strains may also have harmful effects on bees. Fungal growth, and production of antimicrobial compounds and mycotoxins depends on many factors, including temperature, water activity, pH, and light conditions. Furthermore, mycotoxin production is often stain-dependent (Beuchat, 1983; Mannaa & Kim, 2017). Sequence-based studies exploring the composition and function of beebread mycobiome, including the genera *Aspergillus*, *Alternaria*, *Cladosporium*, *Botrytis*, *Mucor*, *Penicillium*, and *Rhizopus*, identify fungi to the genus or species level. Herein presented results show the metabolic diversity of the different strains of *T. purpureogenus* and their associated properties, including toxicity. Thus, the genus *Talaromyces* may be present as a mixture of species/strains with different metabolic activities, as in the case of bee-related *Aspergillus* spp. These fungi are present in the hive as an assemblage of toxigenic and atoxigenic strains, and some of them are opportunistic pathogens (Becchimanzi & Nicoletti, 2022). Strains of other genera commonly found in bee bread, such as *Alternaria* and *Penicillium*, are also considered to be beneficial but can produce both antimicrobial molecules and mycotoxins that are lethal to bees (Frisvad et al., 2004; Lou et al.,

2013). I assume that the balanced composition of the fungal strains can be an important factor for the health of bees and the whole colony.

In conclusion, bee-associated fungal strains are still an unexplored source of biologically active compounds that can benefit bee health research and natural product discovery. Herein, the promising antibacterial and antiviral/protective activity of extracts B18 and B19 against *Paenibacillus* spp, FCoV, FCV, influenza A viruses, and CBPV (in the case of extract B18) is highlighted. According to the results of this work, it would be conceivable that the substances obtained by fermentation from filamentous fungi could be used as feed additives prophylactically or therapeutically against bacterial and viral infections in honey bees.

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V Published work

Article

Screening of Honey Bee Pathogens in the Czech Republic and Their Prevalence in Various Habitats

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Simple Summary: Worldwide, mass losses of honey bee colonies are being observed more frequently in recent times. Except for the overuse of pesticides, one of the main reasons for high honey bee colony collapse is diseases. For this reason, nationwide screening of common pathogens involving viruses, bacterial, fungal, and protozoa pathogens was performed in three different types of habitat including agroecosystems, towns, and national parks. The most frequent eukaryotic pathogens were Trypanosomatids and *N. ceranae* and in the case of viruses DWV-A and ABPV. In addition, the association between the occurrence of particular pathogens and winter colony losses was found. Although the differences in mortality between individual habitats were not significant, results of this study suggest a significant correlation between DWV-B and DWC-C occurrence and mortality of bee colonies, despite their relatively low occurrence.

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Abstract: Western honey bee (*Apis mellifera*) is one of the most important pollinators in the world. Thus, a recent honey bee health decline and frequent honey bee mass losses have drawn attention and concern. Honey bee fitness is primarily reduced by pathogens, parasites, and viral load, exposure to pesticides and their residues, and inadequate nutrition from both the quality and amount of food resources. This study evaluated the prevalence of the most common honey bee pathogens and viruses in different habitats across the Czech Republic. The agroecosystems, urban ecosystems, and national park were chosen for sampling from 250 colonies in 50 apiaries. Surprisingly, the most prevalent honey bee pathogens belong to the family Trypanosomatidae including *Lotmaria passim* and *Crithidia mellificae*. As expected, the most prevalent viruses were DWV, followed by ABPV. Additionally, the occurrence of DWV-B and DWV-C were correlated with honey bee colony mortality. From the habitat point of view, most pathogens occurred in the town habitat, less in the agroecosystem and least in the national park. The opposite trend was observed in the occurrence of viruses. However, the prevalence of viruses was not affected by habitat.

Keywords: *Apis mellifera*; deformed wing virus; screening; trypanosomatids

1. Introduction

The western honey bee (*Apis mellifera*) is one of the most important pollinators of many agricultural crops and wild plants worldwide. Overall, annual economic evaluation of the pollination service was quantified in 2005 to 153 billion euros, representing a yield of about 10% of global agriculture production [1]. Considering the ecological and economical importance of pollination, the widespread honey bee colony losses are a worrying phenomenon [2]. Researchers have found many factors that are a potential cause of honey bee collapse including viral [3], fungal [4], and bacterial diseases [5] together with the use of pesticides [6]. Other factors leading to the collapse of honey bee colonies are parasites,

chemical treatments (amitraz, tau-fluvalinate, coumaphos, antibiotics), nutritional stress (pollen monodiete), and others [7,8]. Some stressors act synergistically such as *Nosema apis* and some pesticides [9]. The collapse of honey bee colonies is thus probably caused by combinations of multiple factors. Therefore, it is necessary to look at and deal with the health of honey bee colonies comprehensively [10].

Recently, however, viral diseases have largely contributed to bee colony losses. The most common and most dangerous virus is a deformed wing virus (DWV). This single-stranded RNA virus is a member of *Iflaviridae* [11] and creates highly genetically heterogeneous forms known as quasispecies, which can exist as several master variants [12]. One of them is type A (DWV-A), which has been attributed to the global decline in honey bees [13–15]. Another variant is type B (DWV-B), known as Varroa destructor virus-1 (VDV-1) [16,17], since it was isolated from the Varroa mite for the first time [18]. The third master variant is type C (DWV-C). However, its impact on honey bees is still unclear [12]. Other common honey bee viruses are slow bee paralysis virus (SBPV), acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), black queen cell virus (BQCV), sacbrood virus (SBV), Lake Sinai virus (LSV), and Macula-like virus (MLV) [19]. Their increasing distribution is mainly due to the ubiquity of the *Varroa destructor* mite, which serves as a vector and transmits viruses [10], both directly on honey bees and indirectly on other insect pollinators [20].

Another dangerous pathogen is the bacteria *Paenibacillus larvae* causing the disease called American foulbrood (AFB). Several genotypes (ERIC I-V) of this bacteria are known, and each has its specific properties such as virulence or distribution area [21]. American foulbrood is one of the most infectious honeybee diseases spread worldwide [22]. In some countries (USA, Canada, Argentina), it is allowed to use antibiotics against AFB. However, antibiotic treatment can only mitigate the symptoms but not eliminate the disease. Moreover, the antibiotics leave residues in the honey and their use in beekeeping is prohibited in many countries [23]. Given that the spores of this bacterium are very resilient and remain viable for more than 35 years, the only effective provision against the spread of *P. larvae* is to burn the infected hives together with combustible beekeeping equipment. It is essential to monitor infected habitats and their surroundings for a long time [5].

The bacterium *Melissococcus plutonius*, the causal agent for European foulbrood, has a similar infection course and method of control. It often appears together with other bacteria, so-called secondary invaders. This pathogen causes great problems, especially in the UK and Switzerland [24,25]. However, *M. plutonius* has been recorded in the Czech Republic in 2015 after a long time [26].

Important parasites are also pathogenic fungi *Nosema apis* and *Nosema ceranae,* which cause disease of the digestive tract of adult honey bees. At present, this disease is considered one of the main causes of the collapse of honey bee colonies during the winter period [10,27].

So far, less attention has been drawn to fungal diseases such as chalkbrood disease caused by entomopathogenic fungus *Ascosphaera apis* [28]. It causes mummification of bee larvae in the hive, resulting in weakening the colony and increasing susceptibility to other pathogens. Under suitable environmental conditions, the reproductive potential of the pathogen increases [29]. In some cases, it can even cause the death of bee colonies [30]. In addition, its worldwide distribution and its frequent occurrence make it an economically significant disease on a global scale [31,32].

Recently, of concern is also an infection by parasitic protozoa *Crithidia mellificae* and *Lotmaria passim* belonging to the order Trypanosomatida [33], which were previously considered relatively harmless [34]. However, it turns out that they can cause significant losses of honey bee colonies, especially with co-infection with *Nosema ceranae* [35–37]. Castelli et al. [38] also reported an association between the infected colonies and higher level of *V. destructor* infestation. Furthermore, honey bees have a highly conserved and specialized intestinal microbiome [39] that might be disrupted by trypanosomatids [40]. *L. passim* species has only recently been described [33] and now represents the dominant trypanosomatids species [37], which has already been detected in the Czech Republic [40].

All of the above-mentioned pathogens contribute to the deaths of honey bee colonies. In particular, they have a significant negative effect on the bees' winter generation, which, due to stronger immunity and longevity, ensures the survival of honey bee colonies during winter. However, since the winter generation of bees is weakened, the length of their lives is significantly reduced, which might subsequently lead to honey bee colony losses [41].

To inhibit pathogens within the congenital and social immunity and for the proper development of honey bee brood, the quality of honey bee nutrition represented by pollen is crucial. In particular, its diverse composition with a broader range of biologically active substances significantly contributes to strengthening the bee detoxification capacity [42], immunity, and resistance to overcome some diseases [43] or viral infections [44]. In contrast, the low diversity of food resources can cause malnutrition and, together with the cocktail of pesticides applicable on the fields, can shorten the life of the winter generation of bees. This can disrupt the immune response of bees, which are then more susceptible to pathogens, parasites, and other stressors. This situation occurs more often in intensively cultivated agricultural areas where a significant change in the landscape has been made, leading to a reduction in biodiversity [45]. Very specific are urban areas, which have recently become increasingly popular for beekeeping. These are mainly characterized by a built-up area and high human disturbances. Nevertheless, urban areas also contain parks, gardens, and other seminatural areas, which provide honey bees with continual nectar and pollen flow [46]. Protected areas are represented by a less anthropogenically influenced landscape characterized by a high diversity of vegetation providing rich food resources and a low level of chemical contamination [47].

This study aims to evaluate the prevalence of the main honey bee pathogens in the Czech Republic, depending on different types of habitats representing various anthropogenic burdens as well as to determine the possible impact of individual pathogens and their co-infection on the honey bee colony losses during the winter period.

2. Materials and Methods

2.1. Sampling

Samplings were carried out from selected apiaries placed in different landscapes across the Czech Republic in the fall of 2019. Agroecosystems, urban ecosystems, and national parks were chosen concerning different urban burdens to sample biological material from 250 hives in 50 apiaries (22 apiaries in agroecosystems, 22 apiaries in urban ecosystems, and six apiaries in the national park). From each apiary, five beehives were randomly chosen. Approximately 50 honey bees were collected from the brood frame of each beehive and immediately frozen on dry ice. The samples were stored at -80 °C until processing. All brood frames from the tested colonies were checked for symptoms of bacterial bee brood diseases. The colony losses were assessed in spring 2020 (the percentage of collapsed colonies of the whole apiaries).

2.2. Characterization of Different Types of Habitat

The town habitat in the Czech Republic involves especially built-up area of towns with houses and factories and is affected by increased industrial contamination and high levels of traffic. Therefore, it represents the highest urban burdens. This habitat also includes town parks and gardens. The agroecosystems are characterized by large areas of fields with agricultural crops, especially monocultures, a high rate of landscape fragmentation and agrochemical contamination. In addition, low diversity of bee food sources as well as short-term availability of food due to intensive agricultural management is typical. National parks, as the most potential honey bee-friendly environment with minimal human disturbance is characterized by flowery meadows, pastures, and forests. Habitat is characterized by an absence of industry, a low degree of landscape fragmentation, and a rich diversity of flowers, which are a good source of food for bees. Agricultural management is possible only through an ecological approach without the use of pesticides.

2.3. Sample Preparation and Nucleic Acid Purification

Samples for RNA (detection of DWV-A, DWV-B, DWV-C, BQCV, CBPV, ABPV, SBV, LSV, MLV) and DNA (detection of *Nosema apis*, *Nosema ceranae*, *Paenibacillus larvae*, *Melissococcus plutonius*, *Ascosphaera apis*, *Crithidia mellificae*, *Lotmaria passim*) purification were collected as a bulk of approximately 250 bees from five hives in each location, frozen in dry ice, and stored at −80 °C. After homogenization in liquid nitrogen, aliquotes for separate RNA and DNA purification were made.

According to the manufacturer's instructions, total RNA was extracted using the TRI Reagent (MRC, Montgomery, OH, USA). Contaminating DNA was removed using the DNA-free TM Kit (Ambion, supplied by ThermoFisher Scientific, Loughborough, UK). BioSpec Nano (Shimadzu, Nakagyo-ku, Kyoto, Japan) was used to quantify RNA (OD260) and to assess sufficient quality (OD260/280 ratio and OD260/230 ratio). cDNA templates were prepared using a Standard Reverse Transcription Protocol (Promega, Madison, WI, USA) and OligodT primer and stored at −20 °C until use.

DNA was extracted using a modified CTAB method. Homogenized tissue was resuspended in CTAB buffer (2% CTAB, 100 mM Tris pH 8.0, 20 mM EDTA pH 7.8, 1.4 M NaCl) with 1% β-mercaptoethanol and incubated at 65 °C for 10 min. The solution was extracted with 500 µL chloroform:isoamylalcohol (24:1) and precipitated in 250 µL of 2-propanol at −20 °C for 30 min. After washing with 1 mL of 70% ethanol, the pellet was resuspended in 150 µL of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 7.8) and stored in 4 °C until use.

2.4. PCR Conditions

The RT-PCR (detection of DWV-A, DWV-B, DWV-C, BQCV, CBPV, ABPV, SBV, LSV, MLV) was performed on the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, supplied by ThermoFisher scientific, Loughborough, UK) using Power SYBR® Green PCR Master Mix (Applied Biosystems, supplied by ThermoFisher Scientific, Loughborough, UK) in a 96-well reaction plate using parameters recommended by the manufacturer (2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s 95 °C, 1 min of 60 °C, 15 s at 95 °C, 1 min at 60 °C, and 15 s at 95 °C). The no-template controls were included. Positive samples were considered a true positive using a Ct cutoff of 36 cycles. The specificity of amplification was determined by dissociation curve analyses and sequencing of randomly selected positive samples. The sequence of the primer, orientation, annealing temperature, and references are shown in Table 1.

The PCR (detection of *Nosema apis*, *Nosema ceranae*, *Paenibacillus larvae*, *Melissococcus plutonius*, *Ascosphaera apis*, *Crithidia mellificae*, *Lotmaria passim*) was performed on the Eppendorf Mastercycler PRO system (Eppendorf, Hamburg, DE) in 25 µL volume containing 1× PPP Master Mix (Top-Bio, Vestec, Czech Republic), 10 pmol each forward and backward primer, and 2 µL of DNA template using the following cycling conditions: denaturation at 95 °C for 5 min, 40 cycles of 30 s 95 °C, 45 s of TA, 1 min at 72 °C; and a final extension at 72 °C for 10 min. PCR products were visualized by 1.5% agarose gel electrophoresis and stained with ethidium bromide solution (Merck Life Science, Darmstadt, Germany). The specificity of amplification was determined by sequencing randomly selected positive samples. The sequence of the primer, orientation, annealing temperature, and references are shown in Table 1.

Table 1. Primers for PCR analysis.

Gene	Sequences 5′-3′	TA [°C]	Reference
Nosema apis	F: GGGGGCATGTCTTTGACGTACTATGTA R: GGGGGGCGTTTAAAATGTGAAACAACTATG	62	[48]
Nosema ceranae	F: CGGCGACGATGTGATATGAAAATATTAA R: CCCGGTCATTCTCAAACAAAAAACCG	62	[48]
Paenibacillus larvae	F: GCTCTGTTGCCAAGGAAGAA R: AGGCGGAATGCTTACTGTGT	55	[49]
Melissococcus plutonius	F: GAAGAGGAGTTAAAAGGCGC R: TTATCTCTAAGGCGTTCAAAGG	55	[50]
Ascosphaera apis	F: TGTGTCTGTGCGGCTAGGTG R: GCTAGCCAGGGGGGAACTAA	60	[51]
Crithidia mellificae	F: AGTTTGAGCTGTTGGATTTGTT R: AACCTATTACAGGCACAGTTGC	56	[52]
Lotmaria passim	F: TGACTTGAATTAGCAAGCATGGGATAACA R: CCTTTAGGCTACCGTTTCGGCTTTTGTTGGT	60	[53]
DWV-A	F: CGTCGGCCTATCAAAG R: CTTTTCTAATTCAACTTCACC	60	[54]
DWV-B	F: GCCCTGTTCAAGAACATG R: CTTTTCTAATTCAACTTCACC	60	[54]
DWV-C	F: TACTAGTGCTGGTTTTCCTTT R: ATAAGTTGCGTGGTTGAC	60	[54]
BQCV	F: GGACGAAAGGAAGCCTAAAC R: ACTAGGAAGAGACTTGCACC	48	[48]
CBPV	F: AACCTGCCTCAACACAGGCAAC R: ACATCTCTTCTTCGGTGTCAGCC	60	[55]
ABPV	F: TGAGAACACCTGTAATGTGG R: ACCAGAGGGTTGACTGTGTG	48	[56]
SBV	F: GGATGAAAGGAAATTACCAG R: CCACTAGGTGATCCACACT	48	[56]
LSV	F: CKTGCGGNCCTCATTTCTTCATGTC R: CATGAATCCAAKGTCAAAGGTRTCGT	60	[57]
MLV	F: ATCCCTTTTCAGTTCGCT R: AGAAGAGACTTCAAGGAC	60	[58]

2.5. Statistical Analysis

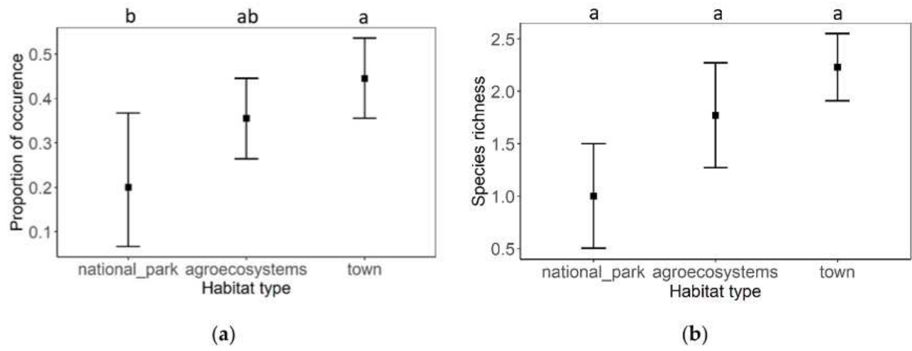
To evaluate whether pathogen occurrence and species richness differ among honey bee colonies and habitat types, we used separate generalized liner mixed-effects models (GLMM) [59]. In the case when species richness was used as dependent variable, GLMM with a Gaussian error distribution was used. When the pathogen occurrence or honey bee mortality rate was used as the dependent variable, binomial error distribution with logit link function was used. In each model, we specify habitat types and pathogen species as fixed factors and the owner of the honey bee colony was used as a factor with a random intercept effect. To compare the means within a particular fixed factor, the Tukey multiple comparison test with Bonferroni adjustment of *p*-values was used. Data were analyzed in the R program (R Development Core Team 2020).

To visualize and test the association between the mortality rate of honey bees and species composition of pathogens, partial canonical correspondence analysis (pCCA) was used with the habitat type as the covariable. We used this type of covariable to eliminate the possible confounding effect of habitat type on the mortality of honey bees regardless of the pathogen species composition. The significance of the canonical axis was tested with a

restricted Monte Carlo permutation test for the time series with 2000 permutations. All ordination analyses were conducted by the statistical software CANOCO, v. 5 [60].

3. Results

The proportion of eukaryotic pathogen occurrence significantly differs between town habitat and national park, whereas the lowest rate of pathogen occurrence has been observed in the national park and the highest in the towns. A moderate rate of pathogen burden has been observed in agroecosystems. However, this habitat did not differ significantly between urban areas or national parks (Figure 1a, Table 2). The species richness of eukaryotic honey bee pathogens did not significantly differ between the tested habitats (Figure 1b, Table 2).



(**a**) (**b**)

Figure 1. (**a**) The proportion of eukaryotic pathogen occurrence in different types of habitats and (**b**) the proportion of eukaryotic pathogen richness in different types of habitats. Black squares represent means and the error bars represent 95% confidence intervals. Significant differences (<0.05) are indicated by different letters.

Table 2. The results of the analysis of deviance (likelihood-ratio test) testing the partial effect of habitat type and pathogen species identity on the species richness and occurrence of pathogens in the honey bee colonies. Likelihood-ratio analysis testing of whether the Akaike information criterion (AIC) of the full model significantly increased after a particular explanatory variable was excluded from the model.

	Df.	AIC	LRT	Pr (Chi)
		Dependent variable: species occurrence		
Full model		243.68		
Eukaryote	4	332.25	96.570	<0.0001
Habitat	2	246.76	7.081	0.02899
Full model		398.26		
Virus	9	494.96	114.695	<0.0001
Habitat	2	396.69	2.423	0.2977
		Dependent variable: number of eukaryotic species		
Full model		156.78		
Habitat	2	157.23	4.453	0.107
		Dependent variable: number of virus types		
Full model		181.88		
Habitat	2	180.52	2.642	0.267

In all types of habitat, the same species of eukaryotic pathogens dominated. In all cases, the most dominant species were *L. passim* and *N. ceranae*, followed by *C. mellificae*, and the lowest occurrence rate had *M. plutonius* and *P. larvae*. No clinical symptoms of

bacterial brood diseases were observed. In contrast, *A. apis* and *N. apis* were not detected at all (Figure 2).

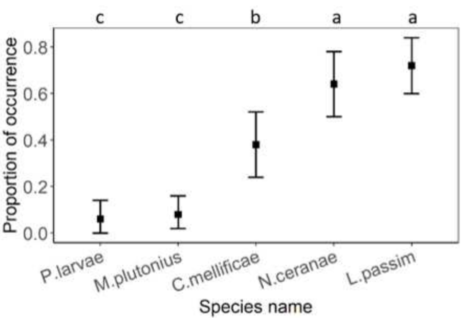


Figure 2. The comparison of proportion of eukaryotic pathogen occurrence regardless of habitat. Black squares represent means and the error bars represent 95% confidence intervals. Significant differences (<0.05) are indicated by different letters.

In the case of individual habitats, all five tested pathogens were detected in a town habitat. The most prevalent pathogens were *L. passim* and *N. ceranae*, followed by *C. mellificae*. Bacteria *P. larvae* and *M. plutonius* only had a low prevalence. The most dominated species in the agroecosystems were *N. ceranae*, *L. passim*, and *C. mellificae*. *M. plutonius* occurred significantly less and *P. larvae* were not detected at all. In the case of national parks, only *L. passim* and *N. ceranae* were detected (Figure 3).

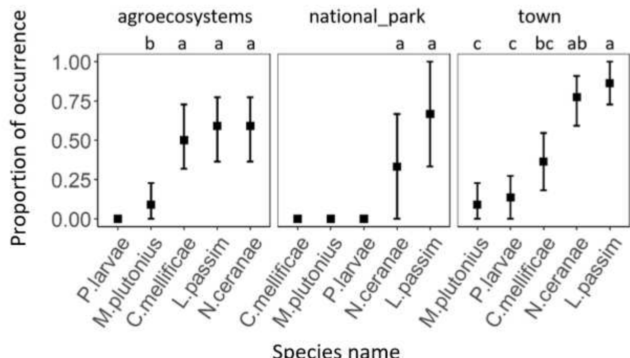
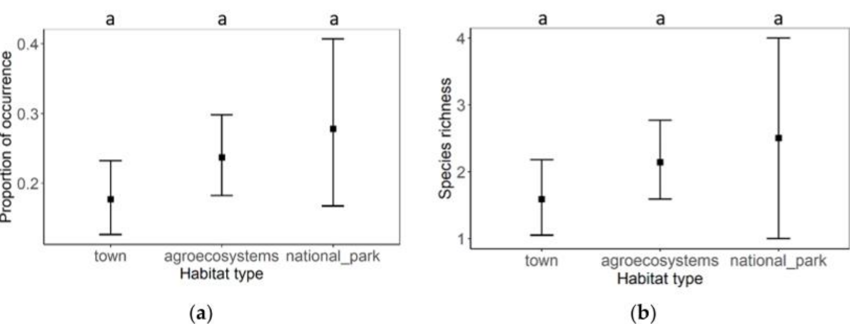


Figure 3. The comparison of proportion of eukaryotic pathogen occurrence within each habitat type. Black squares represent means and the error bars represent 95% confidence intervals. Significant differences (<0.05) are indicated by different letters.

Viral pathogen occurrence and species richness did not significantly differ between individual habitats (Figure 4 and Table 2). Generally, the most abundant viruses were DWV-A and ABPV, followed by DWV-B and LSV. Less frequent viruses were MLV, SBV, CBPV, DWV-C, and BQCV (Figure 5). A similar pattern was observed in all types of habitats. Only DWV-A dominated in the agroecosystems (Figure 6).



(a) (b)

Figure 4. (**a**) The proportion of viral pathogens occurrence in different types of habitats and (**b**) comparison of species richness of viral pathogens between different types of habitats. Black squares represent means and the error bars represent 95% confidence intervals. Significant differences (<0.05) are indicated by different letters.

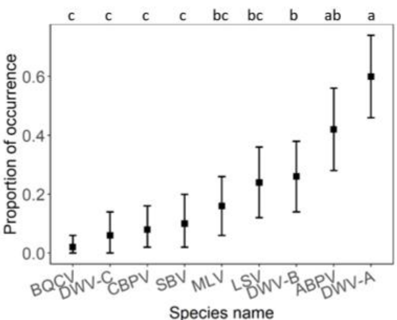


Figure 5. The comparison of proportion of viral pathogen occurrence regardless of habitat. Black squares represent means and the error bars represent 95% confidence intervals. Significant differences (<0.05) are indicated by different letters.

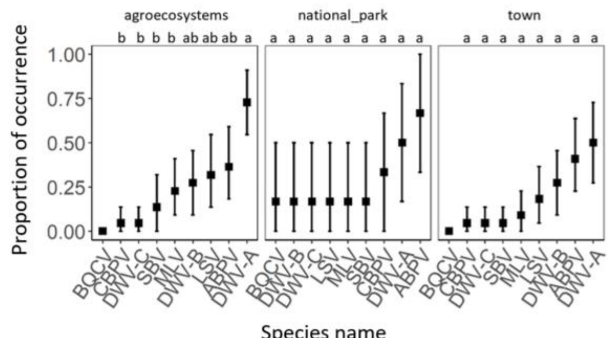


Figure 6. The comparison of proportion of viral pathogen occurrence within each habitat type. Black squares represent means and the error bars represent 95% confidence intervals. Significant differences (<0.05) are indicated by different letters.

Differences winter mortality rates in honey bee colonies between habitats were not statistically significant (Figure 7) due to a small number of samples from national parks and high confidence interval from the data. However, the average winter mortality in

town (24.51%) and agroecosystem (21.50%) habitats were twice as high as in national parks (11.11%).

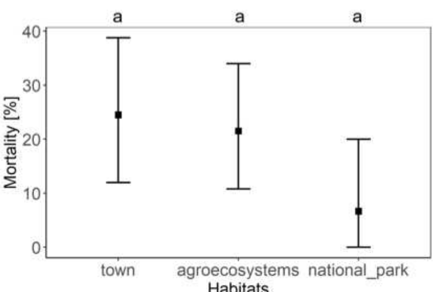


Figure 7. The comparison of honey bee winter mortality rate according to type of habitat. Black squares represent means and the error bars represent 95% confidence intervals.

Based on the results of pCCA species, structures of all pathogens (i.e., species composition and their abundances) were significantly associated with honey bee mortality (pseudo-F = 1.8, p = 0.053, test of all canonical axes, R^2 = 3.73%). In the separate pCCA analyses evaluating association only between viruses and honey bee mortality, we found that the assemblage composed only with viruses (pseudo-F = 2.2, p = 0.037, test of all canonical axes, R^2 = 5.28%) had a closer relationship to mortality than the assemblage composed only with eukaryotes (pseudo-F = 0.3, p = 0.881, test of all canonical axes, R^2 = 0.80%). The pCCA diagram revealed that the closest association with honey bee mortality was shown by DWV-C and DWV-B viruses (Figure 8).

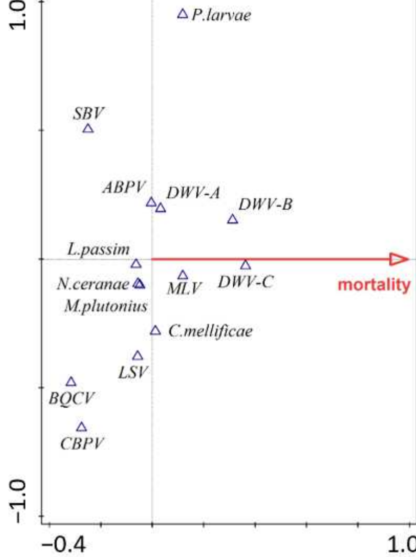


Figure 8. Partial canonical correspondence analysis biplot with the habitat type as a covariable showing the strength of the association of individual pathogens with the mortality rate.

4. Discussion

In the study, the prevalence of several honey bee pathogens was detected including viruses, fungal, protozoa, and bacterial pathogens on different types of habitats. The most frequently detected pathogens belonged to the family Trypanosomatida, in particular, *Lotmaria passim* (72%) and *Crithidia mellificae* (38%). Both protozoa significantly shorten the life of bees and are therefore thought to cause significant bee colony losses [61]. Of even more concern is that trypanosomatids affect the composition of the symbiotic bacterial taxa of bees [40]. However, little is known about the full extent of the harmfulness and mechanism of pathogenesis of these two pathogens [38,62]. Other studies have shown an even higher risk of trypanosomatids when co-infected with *N. ceranae* [35–37]. In addition, it led to a reduction in immune gene expression [37]. The high incidence of trypanosomatids is similar in other European countries [36,62].

The prevalent pathogen is also *Nosema ceranae* (64%), often associated with colony losses, especially in Mediterranean areas [4,63,64]. However, its occurrence has also been recorded in the temperate zone to a lesser extent [10] and with less impact [65,66]. In this study, *N. ceranae* has not been significantly associated with colony losses (Figure 8). This pathogen occurred independently of the habitat type observed. On the other hand, *Nosema apis* was not detected at all. The decline in *N. apis* and the spread of *N. ceranae* is a well-known and long-lasting trend taking place globally [67–71]. However, the complete absence of *N. apis* in the nationwide screening is a novelty. We attribute this to the displacement of the more aggressive *N. ceranae* due to its higher virulence [68,69]. *Ascosphaera apis* was also not been detected. It is an opportunistic pathogen that occurs in the colony, especially in stressful situations such as thermal discomfort [29]. Higher prevalence was recorded in humid areas, and, for example, in China [72] and northern Thailand [30], the fungal pathogen causes great damage.

Bacterial diseases occurred only to a lesser extent and only in urban areas (*P. larvae* and *M. plutonius*) and agroecosystems (*M. plutonius*). They did not occur in the national parks at all. *P. larvae* commonly occurs across the whole Czech Republic, especially in Moravia, and the dominant genotype is ERIC II (80.4%) over ERIC I (19.4%) [73]. The outbreak of European foulbrood caused by *M. plutonius* was observed in 2015 after 40 years in the Czech Republic. Since then, the occurrence persists, but with a very low prevalence [74]. In contrast, in some countries such as England [75], France [76], and Switzerland [77], bacterial disease very often occurs. These two bacterial diseases are very infectious and can cause great economic losses. Therefore, the government often monitors its prevalence, and in many cases, there is an effort to eliminate them through strict rules.

In the case of viral diseases, at least one of the tested honey bee viruses were detected in 74% of cases, while two or more viruses were present in one-third of the tested apiaries. The most prevalent honey bee virus was the deformed wing virus (DWV). There are multiple variants of DWV that include type A [11], type B (Varroa destructor virus-1 (VDV-1) [14,18], and type C [12]. These variants have a different impact on honey bee colonies, and their virulence is not clear. Whereas some studies claim DWV-A has higher virulence [16,78,79], other studies claim DWV-B has the same or even higher virulence [17,80–82]. Since the variant DWV-B can replicate in Varroa mites, the viral load is usually higher in honey bee tissues than in other DWV variants [78,83]. DWV-C is associated with DWV-A and has been indicated as a contributing factor in overwintering losses of honey bee colonies [78,79]. Our study reports DWV-A as the most frequent variant (60%) in the Czech Republic (Figure 5). Surprisingly, similar results where variant DWV-A dominated have been reported from the USA [79,83], whereas variant B dominated in Europe [78,80,84]. However, despite their low prevalence, only DWV-B (26%) and C (6%) variants were significantly associated with the overwintering losses (Figure 8). Other authors have also concluded that these variants are associated with winter colony losses [17,85].

The second most prevalent virus was ABPV, which was detected in half of the tested colonies. This virus has commonly been detected in Germany [10], the USA [3], Switzerland [86], and Belgium [87] and its co-infection with DWV is attributed to overwintering

losses [10]. The LSV (24%) virus is also a major concern, especially in the USA [88]. However, its prevalence is also high in Europe [36]. One of the recently identified honey bee viruses is MLV (16%), which is associated with the mite *V. destructor* [89]. However, its virulence and impact on honey bees are still unclear [90]. Its high prevalence has been observed in France [89], Belgium [36], and Syria [91]. The occurrence of SBV (10%), CBPV (8%), and BQVC (2%) was only minor, especially in urban areas and agroecosystems. The presences of these viruses were not significantly related to the decline of honey bee colonies in the Czech Republic.

The lowest occurrence of eukaryotic pathogens was detected in the national parks, higher occurrence in the agroecosystems, and the highest occurrence in town habitats (Figure 1). This probably corresponds with a high density of bee colonies in the landscape [92] because the number of bee colonies per km^2 in the Czech Republic is one of the highest in the world (>8 honey bee colonies/km^2) [93]. According to these results, Taric [94] also found a higher parasitic burden in commercially kept colonies than traditionally kept colonies, which are mostly situated in natural areas. The richness of individual pathogens was in the same trend, where only two eukaryotic pathogens were present in the national parks. At the same time, four of them occurred in the agroecosystems and five in the towns.

The opposite trend was observed for viruses. All nine tested viruses were present in the national parks, while in agroecosystems and towns, there were eight species. However, these differences were not statistically significant. The study shows that the occurrence of honey bee pathogens, and especially viruses, did not differ between the tested habitats. In addition, the viruses also spread quickly among other species of wild pollinators, which can cause problems with species composition and affect trophic bonds and ecosystem stability [20,84,95].

Differences in the mortality between habitats were not statistically significant. The results were not significant probably due to the low number of samples from the national parks. One of the reasons for colony mortality in national parks is probably due to the high prevalence of viruses as in other habitats (DWV-B and DWV-C), which were associated with colony mortality. The next issue is the trading of bee queens or whole colonies and the migratory management of colonies [96]. This is connected with colony density, which is usually lower in natural parks. This might be another reason for lower honey bee eukaryotic pathogen occurrence in natural parks. At localities with a high bee density, bee colonies cannot avoid sharing food resources, which represent hotspots of infections [97].

5. Conclusions

The most prevalent eukaryotic pathogens in the population of *A. mellifera* in the Czech Republic were *L. passim* and *N. ceranae*, followed by *C. mellificae*. This trend was valid in all types of monitored habitats. In contrast, *P. larvae* and *M. plutonius* were detected only sporadically. *N. apis* and *A. apis* were not detected at all.

The most prevalent viruses were DWV-A and ABPV in all types of tested habitats. On the other hand, BCQV, SBV, and DWV-C were the least prevalent, except in national parks, where the occurrence of all the monitored viruses was relatively uniform.

Of all the monitored eukaryotic and viral pathogens, only DWV-C and DWV-B were significantly associated with colony mortality.

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Article

Extracts of *Talaromyces purpureogenus* Strains from *Apis mellifera* Bee Bread Inhibit the Growth of *Paenibacillus* spp. In Vitro

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Abstract: Honey bees coexist with fungi that colonize hive surfaces and pollen. Some of these fungi are opportunistic pathogens, but many are beneficial species that produce antimicrobial compounds for pollen conservation and the regulation of pathogen populations. In this study, we tested the in vitro antimicrobial activity of *Talaromyces purpureogenus* strains isolated from bee bread against *Paenibacillus alvei* (associated with European foulbrood disease) and three *Aspergillus* species that cause stonebrood disease. We found that methanol extracts of *T. purpureogenus* strains B18 and B195 inhibited the growth of *P. alvei* at a concentration of 0.39 mg/mL. Bioactivity-guided dereplication revealed that the activity of the crude extracts correlated with the presence of diketopiperazines, a siderophore, and three unknown compounds. We propose that non-pathogenic fungi such as *Talaromyces* spp. and their metabolites in bee bread could be an important requirement to prevent disease. Agricultural practices involving the use of fungicides can disrupt the fungal community and thus negatively affect the health of bee colonies.

Keywords: *Apis mellifera*; honey bee; fungi; bee bread; *Talaromyces*; antimicrobial activity; biocontrol; natural product

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1. Introduction

The reproduction of most flowering plants and ~30% of all crops is dependent on pollination [1,2]. The estimated value of pollinators to the agricultural economy was USD 164 billion in 2009 [3] and now stands somewhere between USD 235 and USD 577 billion annually [4,5]. This value increases in line with crop production, and so does our dependence on pollinators for the maintenance of food security [2,4,6,7]. However, there has been a recent, dramatic decline in natural pollinator abundance and diversity [8,9]. Accordingly, managed pollinators such as honey bees (*Apis mellifera*, Linné 1758) (Hymenoptera: Apidae) play a key role in agroecosystems by augmenting wild pollinators and thus ensuring crop quality and yield stability. However, an array of biotic and abiotic stressors associated with agricultural intensification, parasites, and pathogens negatively affect the health of managed bees [10–12]. The deterioration in bee health results in the loss of colonies, particularly in the northern hemisphere [13–15]. Optimizing the health of individual bees is necessary to improve colony fitness, correlating directly with their benefits to humans, including pollination services as well as the production of honey.

Microorganisms that colonize animals (topically and internally) and their environments often fulfill essential functions [16]. The microbial communities associated with honey bees influence host metabolism, health, and stress tolerance [17–22]. Research has focused on the conserved core gut microbiome, which is dominated by nine clusters of bacterial species that develop 4–6 days after enclosure [23] and are present in almost all individuals regardless of geographic location or season [24–27]. In contrast, less attention has been paid to the composition and function of in-hive microbial communities that colonize the bee cuticle, hive surfaces, and food stores [28]. These consist of bacteria, yeast, and several genera of filamentous fungi that form the core mycobiome of pollen and bee bread, including *Aspergillus*, *Cladosporium*, *Botrytis*, *Penicillium*, *Alternaria*, *Mucor*, and *Rhizopus* [29]. Fungi are important for bee nutrition because they participate in pollen fermentation and its transformation into bee bread [30]. They also break down toxins [31] and confer resistance to fungal pathogens such as *Ascosphaera apis* (Maassen ex Claussen, L.S. Olive, and Spiltoir, 1955), the cause of chalkbrood disease, by producing antifungal compounds [32–34]. Some strains also reduce the viral load in the colony [35,36] and act as probiotics to promote the growth of symbiotic bacteria [37].

The genus *Talaromyces* (Benjamin, 1955) (Eurotiales: Trichomaceae) is a source of natural compounds with applications in medicine and the food industry (as producers of *Monascus*-like pigments) [38–41]. These fungi are known to antagonize plant pathogens and to be associated with insects [42]. The interactions within such complex ecological relationships are mediated by specialized microbial metabolites, and *Talaromyces* species produce diverse natural products that can be used for biological control [38,39].

Only a few studies have reported the presence of *Talaromyces* spp. associated with bees [34,36,42,43]. Here, we characterized *Talaromyces* strains recently isolated from honey bee bread [36] using a combination of colony morphology, DNA barcoding, and phylogenetic analysis. Bee-related *Talaromyces* strains have already been shown to inhibit human pathogens (*T. versatilis*) [43], mammalian and bee viruses (*T. purpureogenus*) [36], and fungal bee pathogens (*T. scorteus* and *T. dendriticus*) [34]. We tested the activity of organic crude extracts against the *Aspergillus* species (Eurotiales: Aspergillaceae) that cause stonebrood disease [44] and the bacterial opportunistic pathogen *Paenibacillus alvei* (Bacillales: Paenibacillaceae). This spore-forming bacterium is often isolated from colonies affected by European foulbrood disease (EFB), which is caused primarily by *Melissococcus plutonius* [45,46]. Both *Aspergillus* and *P. alvei* are also present in healthy colonies [47,48], but stonebrood disease is prevented by hygienic beekeeping practices and EFB has, until recently, been prevented by the use of oxytetracycline [49]. This is becoming less effective due to the ban on apiary antibiotics in many countries and the emergence of antibiotic-resistant pathogens, leading to heavy EFB infestations that often require the destruction of the colony to avoid disease spread [50]. The same measures might be needed for colonies severely infected with stonebrood disease, due to the health risk for beekeepers and consumers [51]. Defining the function of bee bread fungi such as *Talaromyces* strains and their metabolites in the control of these diseases would facilitate the development of new and more effective honey bee health protection strategies.

2. Materials and Methods

2.1. Bee Bread Collection and Fungal Cultivation

The fungal strains were isolated from *A. mellifera* bee bread collected in Kamenny Malikov, Czech Republic (KM Zirovnice; 49°12′51.533′′ N, 15°7′5.129′′ E) in March/April 2019 as previously described [36]. Briefly, hive frames containing stored pollen of various ages were cut out, and bee bread from 10 randomly selected cells was suspended in 0.9% NaCl containing 1% Tween-80 (Sigma-Aldrich, St. Louis, MO, USA). The suspension was then inoculated onto potato dextrose agar (PDA, VWR International, Radnor, PA, USA) at 25 °C. Fungi were subcultured several times until axenic isolates were obtained and identified. The *Talaromyces* strains were deposited in the Fraunhofer strain collection (EXT111748–EXT111754); other fungal genera were excluded from this study.

To obtain spore suspensions, pure colonies were washed with 2 mL of 0.2% agar (Carl Roth, Karlsruhe, Germany) containing 0.05% Tween-80 [52], filtered through three layers of miracloth and stored at 4 °C. Three 1-µL drops were inoculated onto solid media: Czapek yeast autolysate (CYA) medium, malt extract agar (MEA), yeast extract supplemented (YES) medium, creatine sucrose agar (CREA) (according to [52]), and Sabouraud's dextrose agar (SDA)—30 g/L Sabouraud dextrose broth (Merck Millipore, Burlington, MA, USA) and 15 g/L agar (Carl Roth, Karlsruhe, Germany). After 7 or 10 days, colonies were transferred to liquid malt peptone (MP) medium—30 g/L malt extract (Thermo Fisher Scientific, Dreieich, Germany), 5 g/L mycological peptone from meat (Carl Roth, Karlsruhe, Germany) as previously described [36]. The workflow is summarized in Figure 1. Images of fungal colonies were captured using an EOS 450D camera (Canon, Tokyo, Japan) and edited in GIMP ver. 2.10.34 [53].

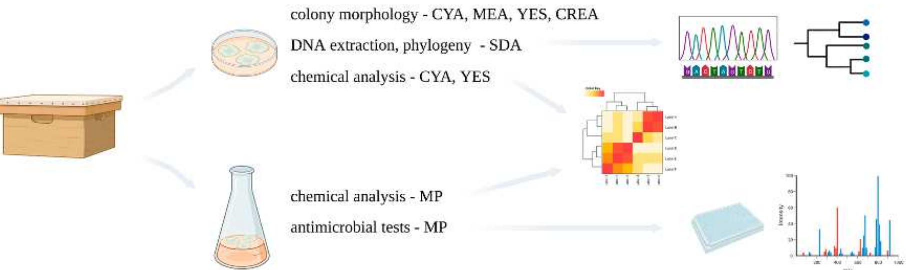

Figure 1. Workflow showing the use of cultivation media prior to colony morphology assessment, molecular barcoding, and phylogenetic analysis (top right), chemical analysis (cosine similarity, center), antimicrobial assays and bioactivity-guided dereplication (bottom right). Figure created with BioRender.com.

2.2. Extract Preparation for Chemical Analysis and Antimicrobial Tests

Extracts were prepared from cultures grown on solid CYA and YES medium for 10 days [52]. Briefly, three plugs were cut from the center of single colonies and transferred to 2 mL screw cap vials. We added 0.8 mL ethyl acetate containing 1% formic acid and incubated in an ultrasonic bath (35 kHz) for 30 min [52]. The liquid phase was transferred to a fresh vial and evaporated under nitrogen, and the residues were redissolved in 40 µL methanol and stored at 4 °C. The fungal strains were also incubated in liquid MP medium and were lyophilized and extracted in methanol as previously described [36]. The crude extracts were redissolved in methanol to a concentration of 100 mg/mL and were stored at –20 °C. Before UHPLC-HR-MS analysis, all samples were centrifuged (8000× *g*, 1 min, room temperature), and 30–40 µL was transferred to 2-mL HPLC vials with glass micro-inserts.

2.3. Molecular Barcoding and Phylogenetics

We collected mycelia from 7-day-old colonies grown on SDA using a sterile scalpel and homogenized the tissue under liquid nitrogen with a mortar and pestle. The ground tissue (50–80 mg) was mixed with 490 µL 2 × CTAB buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris of pH 8) preheated to 60 °C. We then added 10 µL of proteinase K (50 mg/mL) and incubated at 60 °C for 1 h on a shaking platform. The lysate was extracted by adding 500 µL 24:1 chloroform:isoamylalcohol, and the aqueous layer (400 µL) containing was transferred to a new 1.5 mL Eppendorf tube. The DNA was precipitated by adding 270 µL isopropanol. After centrifugation (14,000× *g*, 10 min, room temperature), the pellet was washed with 1 mL ice-cold 70% ethanol and dried in a vacuum concentrator for 10 min. The DNA was dissolved in 20 µL of TE buffer with 2 µL of RNase A (10 mg/mL) for 3 h at 37 °C. Samples were then stored at –20 °C. The concentration and quality of DNA was determined using a NanoDrop UV spectrophotometer and by 1% agarose gel electrophoresis.

To create the barcodes, we amplified four DNA regions: β-tubulin (BenA), calmodulin (CaM), the internal transcribed spacer (ITS), and RNA polymerase II second largest subunit (RBPII), which are often used for the molecular identification and phylogenetic analysis of fungal isolates [52,54] (Table 1). Each 20 μL PCR mix contained 10 μL Taq 2× Master Mix, 0.25 μM of each primer, 100–200 ng of template DNA, and PCR-grade water to top up. The reaction conditions are summarized in Table 2. PCR products were purified using ExoSAP-IT (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions but using modified thermal conditions (37 °C for 35 min, 85 °C for 15 min, and 10 °C infinite hold). The cleaned PCR products were sent with forward and reverse primers for bidirectional sequencing (Eurofins, Val Fleuri, Luxembourg).

Table 1. Primer pairs used for the amplification of the four gene regions.

Target	Name	Orientation	Sequence (5′→3′)	Reference
BenA	Bt2a	For	GGTAACCAAATCGGTGCTGCTTTC	[55]
	Bt2b	Rev	ACCCTCAGTGTAGTGACCCTTGGC	
	Bt1a	For	TTCCCCCGTCTCCACTTCTTCATG	
	Bt1b	Rev	GACGAGATCGTTCATGTTGAACTC	
ITS	ITS1F	For	CTTGGTCATTTAGAGGAAGTAA	[56]
	ITS4	Rev	TCCTCCGCTTATTGATATGC	[57]
CaM	CF1	For	GCCGACTCTTTGACYGARGAR	[58]
	CF4	Rev	TTTYTGCATCATRAGYTGGAC	
RBPII	5F2	For	GGGGWGAYCAGAAGAAGGC	[59]
	7CR	Rev	CCCATRGCTTGYTTRCCCAT	

Table 2. PCR thermal profiles for the used primers.

	ITS and CaM	Ben1A and Ben2A	RBPII
Initial denaturation	95 °C/5 min	94 °C/5 min	95 °C/5 min
Denaturation	94 °C/45 s	94 °C/45 s	94 °C/45 s
Annealing	55 °C/45 s	50 °C/45 s	52 °C/45 s
Extension	72 °C/1 min	72 °C/1 min	72 °C/1 min
Final extension	72 °C/7 min	72 °C/7 min	72 °C/7 min

Sequence reads were analyzed and assembled using Geneious v10.2.6, and the assembly consensus sequences of the barcodes were compared to the sequences in the International Sequence Database (INSD) using the GenBank basic local alignment search tool (BLAST) [60]. The search was adjusted for comparison with the type and reference material and the RefSeq Targeted Loci database (available only for ITS sequences). For species assignment based on ITS sequences, we used the recommended identity threshold of ≥97% [61]. Due to the lack of available Ben1A sequences from the type material in the database (December 2022), phylogenetic analysis was carried using the Ben2A, CaM, ITS, and RBPII sequences (Supplementary Table S1).

Sequence alignments were prepared using MUSCLE in Geneious v10.2.6, and single gene trees (Supplementary Figures S1–S5) were constructed using IQtree with default settings [62,63]. From the concatenated alignment, uninformative sites were removed using Gblocks 0.91b [64,65]. Next, we used partition finder in Cipres Science Gateway v3.3 [66] to determine best-fitting models of molecular evolution and partition schemes. Two simultaneous phylogenetic analyses were carried out using MrBayes v3.2 [67–69] with four Markov chains (three heated, one cold) for 10 million generations with a sampling frequency of 1000 and a 25% burn-in. From the resulting trees, a 65% majority-rule consensus tree was constructed and visualized using iTol v6 [70].

2.4. Detection of Talaromyces spp. at Different Time Points and Locations

Samples were collected in July 2019 and April and July 2020 from five apiaries in the South Bohemia region of Czech Republic as described in Section 2.1. The collection sites were Kamenny Malikov village (KM Zirovnice and KM agro) and three apiaries in Ceske Budejovice (CB campus, CB Litvinovice, and CB Kroclov) from urban and suburban areas and the city periphery, respectively. The locations were ranked by anthropogenic influence, focusing on the level of urbanization and the agricultural landscape within ~6 km (Supplementary Figure S6). ITS sequences were used for species assignment.

2.5. UHPLC-HR-MS Analysis and Metabolic Fingerprinting

Samples were processed for metabolic fingerprinting as previously described [71]. Briefly, the samples were fractionated on a 1290 UHPLC system (Agilent, Santa Clara, CA, USA) equipped with DAD, ELSD, and a maXis II (Bruker, Billerica, MA, USA) ESI-qTOF-HRMS. We used a gradient of 0.1% formic acid in water (buffer A) and 0.1% formic acid in acetonitrile (buffer B) at a flow rate of 600 µL/min. The gradient began at 95% A and was held for 0.30 min before a transition to 4.75% A over 18.00 min and 0% A over 18.10 min, with a hold for 22.50 min. The gradient then increased to 95% A over 22.60 min followed by a hold for 25.00 min. The column oven temperature was set at 45 °C, and we used an Acquity UPLC BEH C18 1.7 µm column (2.1 × 100 mm) with an Acquity UPLC BEH C18 1.7 µm VanGuard pre-column (2.1 × 5 mm).

For micro-fractionation [42], extracts were injected into the UHPLC-HR-MS system described above. However, the flow path was changed so that 90% of the flow was diverted to a custom-made fraction collector (Zinsser–Analytik, Frankfurt, Germany) while the rest was analyzed in MS/MS mode in the maXis II. Collision-induced fragmentation at 28.0–35.05 eV was achieved using argon at 10^{-2} mbar. Micro-fractionation assay plates were prepared by injecting 2 or 5 µL of extract. For each injection, 159 fractions were generated and collected on a 384-well plate (fraction length: 7 s). Before screening, the plates were dried under a vacuum using an HT12-II centrifugal concentrator (Genevac, Ipswich, UK) at 35 °C.

For metabolic fingerprinting, raw MS data were processed using DataAnalysis v5.3 (Bruker) including recalibration with sodium formate, followed by RecalculateLinespectra with a threshold of 10,000 and subsequent FindMolecularFeatures (0.5–25 min, S/N = 0, minimal compound length = 8 spectra, smoothing width = 2, correlation coefficient threshold = 0.7). Bucketing was achieved using ProfileAnalysis v2.3 (30–1080 s, 100–6000 m/z, advanced bucketing for 12 s at 5 ppm, no transformation, bucketing basis = H+). We then constructed a cosine similarity heat map.

2.6. Evaluation of Antimicrobial Activity

2.6.1. Antifungal Activity Test

Antifungal activity was tested against three *Aspergillus* species associated with stonebrood disease (*A. flavus* ATCC9170, *A. fumigatus* ATCC10894, and *A. niger* ATCC10549) from Fraunhofer strain collection (STO20519-STO29521) using CLSI M51-A parameters [72]. The *Aspergillus* strains were cultivated on PDA (VWR International, Radnor, PA, USA) for 7 days at 25 °C. The spores were washed by 5–6 mL of 0.05% Tween-80 (Sigma-Aldrich, St. Louis, MO, USA) and filtered through three layers of miracloth to remove the hyphal structures. The inoculum was adjusted to OD_{530} = 0.14–0.46 based on spore morphology [73]. The inoculum was spread evenly over the surface of Mueller–Hinton agar (Carl Roth, Karlsruhe, Germany) using a sterile cotton swab. The depth of the medium was consistent in each Petri dish (4 mm). The extracts from the liquid *T. purpureogenus* strains cultures (Figure 1) were diluted with methanol to 10 mg/mL, and 25 µL of the crude extract was applied to 6 mm cellulose disks and left in the laminar flow cabinet for 30 min to dry. Methanol was used as a control. The disks were placed on the inoculated media and pressed down with sterile forceps. The zones of inhibition were evaluated in triplicate after incubation at 35 °C for 24 h.

2.6.2. Antibacterial Activity Test

We tested the antibacterial activity of the extracts in a dilution series (25–0.39 mg/mL) in triplicate using a micro-broth dilution test against *P. alvei* CCM 2051 provided by the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic) as previously described [74] with modifications. Briefly, extracts were diluted 1:1 with Mueller–Hinton broth, yeast extract, potassium phosphate, glucose, and pyruvate (MYPGP), and 100 μL of the solution was pipetted to the first row of the microdilution 96-well plate. A two-fold dilution series was prepared by transferring the extract solutions (50 μL) to the next rows containing 50 μL of the MYPGP. Dilutions of methanol were included as a solvent growth control. The lyophilized bacteria were cultivated at 37 °C for ~24 or ~48 h on MYPGP agar. The inoculum was diluted in sterile water and adjusted to 0.5 McFarland units using the DEN-1 McFarland densitometer (BioSan, Riga, Latvia). The bacterial suspension was diluted 1:150 with the MYPGP (~10^6 CFU/mL), and 50 μL was added to each well within 15 min. Bacterial growth was measured at 625 nm using an xMark spectrophotometer (Bio-Rad, Hercules, CA, USA). Minimum inhibitory concentrations (MICs) were defined as the lowest concentration of extract or standard that inhibited growth by at least 80% relative to the control (bacterial suspension with no extract/solvent).

2.6.3. Bioactivity-Guided Dereplication

The potency of the *T. purpureogenus* strains extracts was determined using micro-broth dilution assays as previously described [75–77]. Briefly, extracts were screened in a 12-point dilution series (2–0.001 mg/mL) in triplicate. In addition to four *Paenibacillus* strains (*P. lautus* DSM3035, *P. lactis* FH1832, and two unspecified taxa from the Fraunhofer strain collection ST133196 and ST514408 [78]), we also included the control strain *Escherichia coli* ATCC35218 and the type strain *Bacillus subtilis* DSM 10. For all bacteria, the density of the overnight pre-cultures, incubated in cation-adjusted Mueller–Hinton II medium (BD) at 37 °C while shaking at 180 rpm, was adjusted to 5×10^5 cells/mL. The assay plates were set up by pipetting 100 μL of adjusted bacterial suspension in each well, except the medium control wells H01-H05 (blank or "low"). Wells H06-H12 contained bacterial suspensions without extracts or antibiotics (growth control, "high"). On each assay plate, we also assessed the MIC of three reference antibiotics (ciprofloxacin 0.5–0.0002 μg/mL, cefotaxime, and gentamicin both 64–0.03 μg/mL). The dilution series of pure MeOH, the sample triplicate solved in MeOH, and the reference antibiotics was prepared by adding additional 98 μL of bacterial suspension plus 2 μL of the respective test samples to the first well of rows A–G. Hence, the maximal solvent concentration per well was 1%. Next, the 1:2 dilution series were prepared by transferring 100 μL suspension. After the last dilution step in column 12, the remaining 100 μL were discarded. Assay plates were incubated for 18 h at 37 °C and 85% relative humidity, shaking at 180 rpm, before we measured the turbidity at 600 nm on a LUMIstar Omega microplate spectrophotometer (BMG Labtech, Ortenberg, Germany) as a proxy for cell growth. The relative growth inhibition was calculated based on the absorbance units (AU) of the sample and the controls (see above) using the following formula:

$$\text{rel. inh.\%} = 100 * \left[1 - \frac{\text{AU sample} - \text{AU Low}}{\text{AU High} - \text{AU Low}}\right]$$

MICs were defined as above. Extracts with bioactivity observed over at least three dilution steps ($\leq$0.5 mg/mL) were selected for micro-fractionation. Selected extracts were injected twice into our UHPLC-HRMS-MS system (2 and 5 μL μL, see Section 2.5). The dried assay plate was re-screened against *P. lautus* by adding 50 μL of adjusted pre-culture (see above) to each well except column 1 (media blank). Columns 2 and 3 contained no fractions, but a dilution series of gentamicin ranging from 256–0.008 μg/mL. Column 4 contained only bacterial suspension (growth control), while the remaining 320 wells contained 2×159 fractions collected from the two injection and an unfractionated extract

control of the respective volume. Incubation, read-out, and calculation of the relative growth inhibition was carried out as described above.

3. Results

3.1. Colony Morphology

The use of four standard media enabled us to distinguish the fungal strains based on specific morphological traits: size, sporulation color, and pigment production (Figure 2). The size of the cultures on MEA was comparable (Table 3) but differences in red pigment production (*Monascus*-like red pigments) were observed in the fruiting body (B18 > B13 > B195 on reversed MEA and YES) and media (B13 > B195 > B18 on CYA). The production of low levels of acid on CREA was observed only for strain B49 (Figure 2, Table 3).

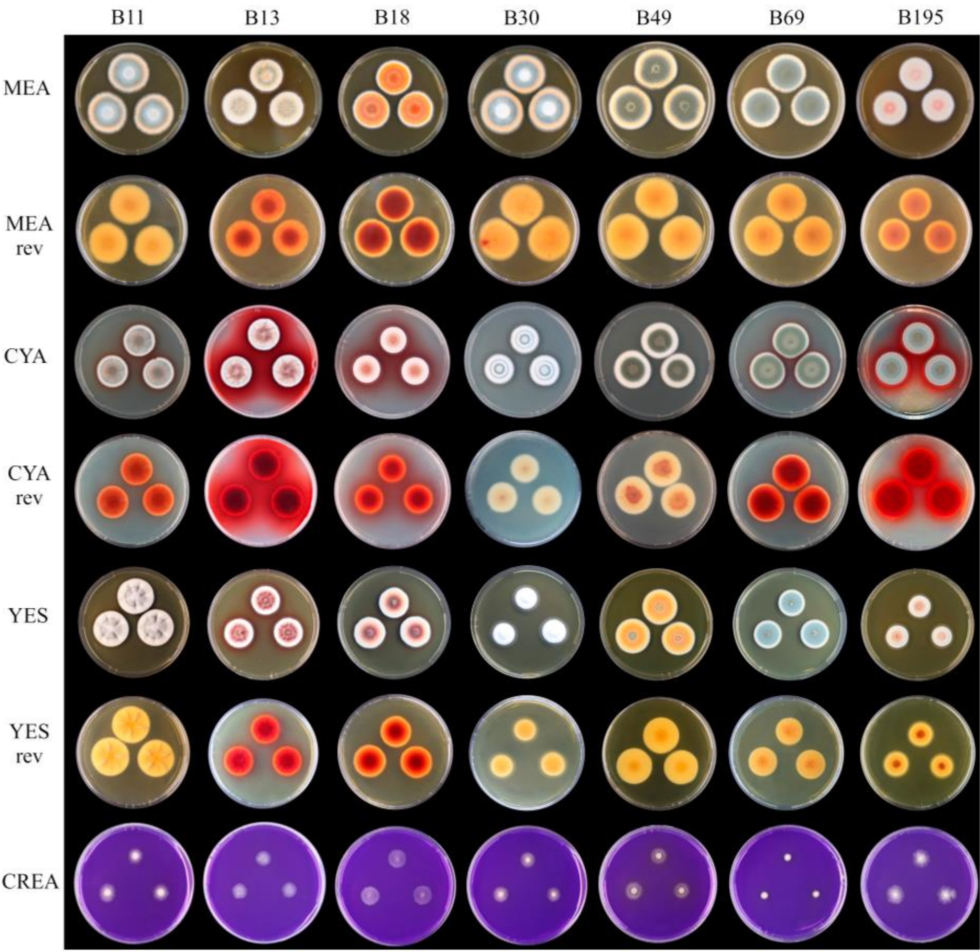

Figure 2. Colony morphology of *Talaromyces purpureogenus* strains isolated from bee bread (columns) when grown on different types of media (rows).

Table 3. Comparison of colony morphology for *Talaromyces purpureogenus* strains isolated from bee bread and species characterization.

Strain	Diameter on CYA (mm)	Diameter on MEA (mm)	Soluble Pigment on CYA	Colony Texture on MEA	Acid Production on CREA	Exudates on MEA
Talaromyces sp. B11	21–25	33–37	Weak red	Velvety to floccose	Absent	Yellow
Talaromyces sp. B13	25–26	25–30	Red	Floccose, wrinkled	Absent	Absent
Talaromyces sp. B18	23–27	35–37	Red	Velvety, floccose	Absent	Orange
Talaromyces sp. B30	21–24	33–40	Absent	Floccose	Absent	Absent
Talaromyces sp. B49	25–30	33–36	Absent	Velvety, floccose	Weak	Colorless
Talaromyces sp. B69	24–28	26–36	Absent	Velvety	Absent	Absent
Talaromyces sp. B195	26–29	27–34	Red	Floccose, funiculous	Absent	Red
T. purpureogenus [54]	20–25	30–45	Red	Velvety, floccose	Absent	–

Our cultivation-dependent method detected isolates of the genus *Talaromyces* in bee bread from three of the five apiaries, but only in a few hives in spring (Table 4). Based on ITS sequencing, we identified *T. purpureogenus* in apiaries in areas with a low anthropogenic influence (CB Kroclov and KM Zirovnice) and two species (*T. purpureogenus* and *T. piceae*) in urban samples (CB campus). We did not detect any *Talaromyces* spp. in the other apiaries (KM agro and CB Litvinovice). Images of the isolates are provided in Supplementary Table S2. Fungal isolates representing other genera were not considered.

Table 4. Presence of *Talaromyces* strains in honey bee bread collected from different sampling sites (apiaries) from 2019 to 2020. The values represent the number of hives where *Talaromyces* strains were found relative to the total amount of hives from which the bee bread was collected.

Apiary	Area	April 2019	July 2019	April 2020	July 2020
KM Zirovnice	Rural	1/6	0/6	1/6	0/6
KM agro	Rural	0/10	0/10	0/10	0/10
CB Kroclov	Periphery	No data	No data	1/3	0/3
CB campus	Urban	No data	No data	2/4	0/4
CB Litvinovice	Suburban	No data	No data	0/4	0/4

3.2. Molecular and Phylogenetic Characterization

We assigned all strains to the species *T. purpureogenus* based on ITS sequence similarity, thus excluding other species in the RefSeq ITS database (Table 5). The sequences were uploaded to the NCBI database (OR192894–OR192900, Supplementary Table S1). By comparing these sequences with the database, we also observed the highest identity (>98%) with *T. purpureogenus* for the other three barcodes (Ben2A, CMD, and RBPII). Only one Ben1A sequence from *Talaromyces* spp. type material was available in the database—*T. stipitatus* (query cover/identity (QC/I) = 88/96.45%; XM_002341495.1). In the standard database, the Ben1A sequences (OR327661–OR327667) showed the highest QC/I to *T. marneffei* (>99/>93.48%; CP045656.1) and less than 90% identity to *T. purpureogenus* isolated from medicinal plants (QC/I = >95/89.65–89.82; HM596783.1) [79].

Table 5. Five sequences with the highest identity in the ITS region based on BLAST analysis. The ITS sequence was identical in all seven strains. QC = query cover, I = identity.

ITS Region Seq.	Database Hits	Score	QC/I [%]	Accession
Talaromyces strains, 577 bp	*T. purpureogenus* CBS 286.36	1000	97/98.93	NR_121529.1
	T. rufus CBS 141834	953	100/96.54	NR_170773.1
	T. thailandensis CBS 133147	942	100/96.19	NR_147428.1
	T. zhenhaiensis CGMCC 3.16102	937	96/96.96	NR_177565.1
	T. aspriconidius CBS 141835	935	97/96.64	NR_170774.1

The resulting phylogeny supports the monophyletic nature of *Talaromyces* and is composed of several subclades containing different members of this genus (Figure 3). General support for the *Talaromyces* clade was high, but several of the deepest clades were only

weakly supported (see Supplementary Figure S1 for numeric bootstrap values). However, the overwhelming majority of shallow clades relevant for the taxonomic placement of our isolates were sufficiently supported to draw firm conclusions.

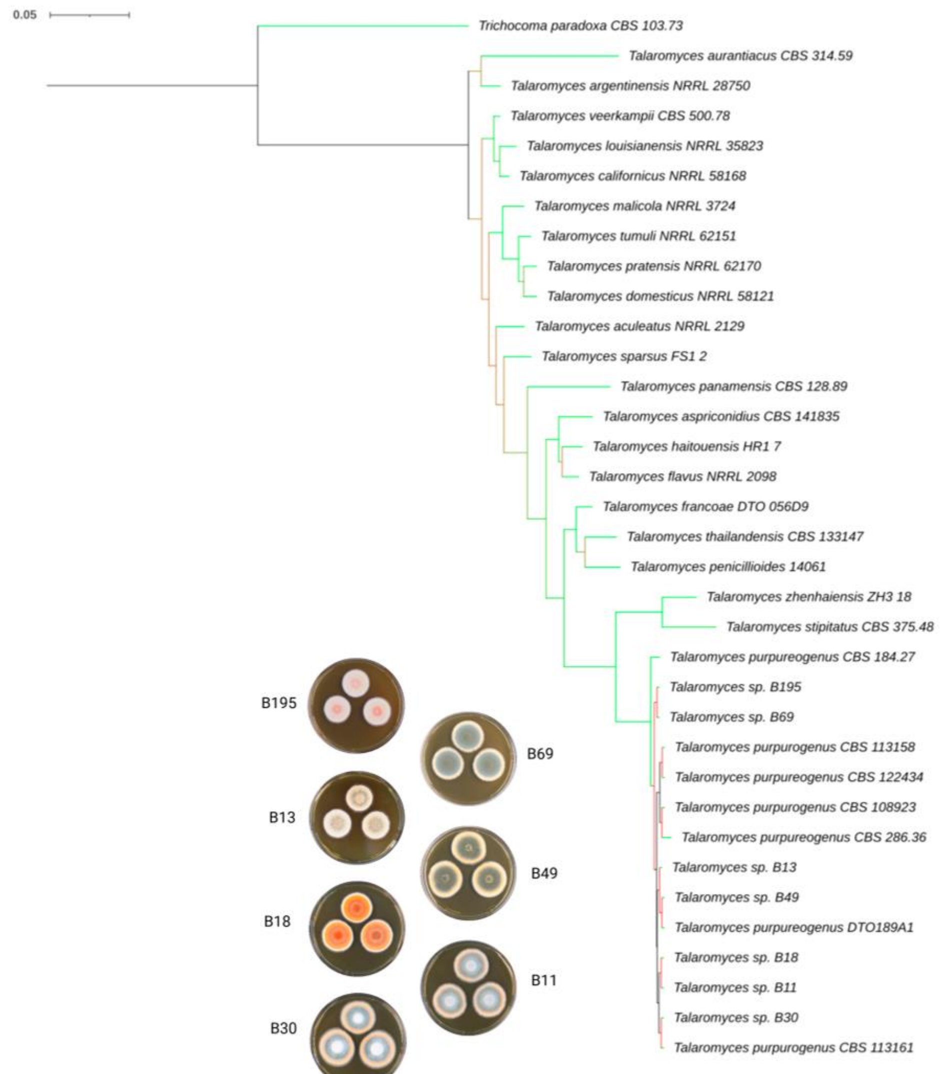

Figure 3. Phylogenetic tree based on ITS, Ben2A, CaM, and RBPII gene regions showing the relationship between the seven newly described *T. purpureogenus* strains and other members of the genus *Talaromyces*. The bootstrap values are indicated using a green–red color model (green = high support and red = low support). The numeric values can be found in Supplementary Figure S1. *Trichocoma paradoxa* was used as the outgroup.

The most ancestral clade within our phylogeny contained two taxa (*T. aurantiacus* and *T. argentinensis*) and was followed by a three-taxon clade (*T. veerkampii*, *T. louisianensis*, and *T. caifornicus*). The next clade contained four taxa (*T. malicola*, *T. tumui*, *T. pratensis*,

and *T. domesticus*) with others splitting off subsequently. The first taxon to split off was *T. aculeatus*, followed by *T. sparsus* and *T. panamensis*, then a clade containing *T. aspriconidius*, *T. haitouensis*, and *T. flavus*, and another with *T. francoe*, *T. thalandensis*, and *T. penicilloides*. The remaining taxa included in our analysis formed the two youngest clades. The first contained two taxa (*T. zhenhaiensis* and *T. stipitatus*) and was placed as a sister to a clade containing all our isolates and different strains of *T. pupurogenus* (CBS 184.27, CBS 113158, CBS 122434, CBS 108923, CBS 286.36, CBS 113161, and DTO189A1). This larger clade, containing the *T. zhenaiensis*/*T. stipitatus* clade plus the *T. purpureogenus* clade, received substantial support (99%). This also applied to both included subclades, which received 100% support for their monophyletic composition. Similar topologies of our isolates in close proximity to *T. purpureogenus* were consistently recovered across all single-gene trees (Supplementary Figures S2–S5). Overall, our phylogenetic analysis supports the assignment of our isolated fungi to the species *T. purpureogenus* as suggested by our ITS DNA barcoding data (Table 5).

3.3. Metabolic Fingerprinting

The extracts showed a broad distribution of compounds over the polarity range (Supplementary Figures S7–S9). The pairwise cosine similarities of all samples were plotted as a heat map (Figure 4). The main grouping correlated with the solid and liquid media, as expected. The clustering order in the solid media was less influenced by the medium (YES and CYA) and the metabolic features of the solid medium controls were therefore very similar (cosine 0.93). The medium controls and strains B11, B13, B30, and B195 clustered in pairs (consisting of one sample each from CYA and YES). Strains B49 and B69 were merged into one cluster. Strains B18 and B195 clustered together in YES, but the metabolic profile of B18 on CYA was distinct from the other strains in this cluster.

In liquid media, the highest similarity in metabolic features was observed between two pairs – strains B30 and B49 (0.91) and strains B11 and B69 (0.83). Whereas all five strains (B11, B13, B30, B49, and B69) were merged into one cluster, strains B18 and B195 were clustered more closely (0.60) and the metabolic features were more distant from those of the other strains. The clustering order of the strains and the corresponding pairwise cosine similarities are summarized in Supplementary Table S3.

3.4. Antimicrobial Activity and Bioactivity-Guided Dereplication

The crude extracts showed no ability to inhibit the growth of any of the *Aspergillus* species (no zone of inhibition) at a concentration of 0.25 mg of per disc. Extracts B195 and B18 inhibited the growth of *P. alvei* at an MIC of 0.39 mg/mL, whereas extracts B11 and B69 showed activity only at higher concentrations, with MICs of 6.25 and 3.13 mg/mL, respectively (Table 6).

Table 6. Minimal inhibitory concentrations of crude methanol extracts from *T. purpureogenus* strains tested at 0.001–2 mg/mL against *B. subtilis*, *P. lautus*, and *P. lactis*, and at 0.39–6.25 mg/mL against *P. alvei* (* using different protocol).

Strains	*B. subtilis*	*P. alvei* *	*P. lautus*	*P. lactis*
B11	>2.00	6.25	>2.00	2.00
B13	>2.00	>2.00	>2.00	>2.00
B18	>2.00	0.39	0.50	0.50
B30	>2.00	>2.00	>2.00	2.00
B49	>2.00	>2.00	>2.00	>2.00
B69	>2.00	3.13	2.00	>2.00
B195	2.00	0.39	2.00	2.00

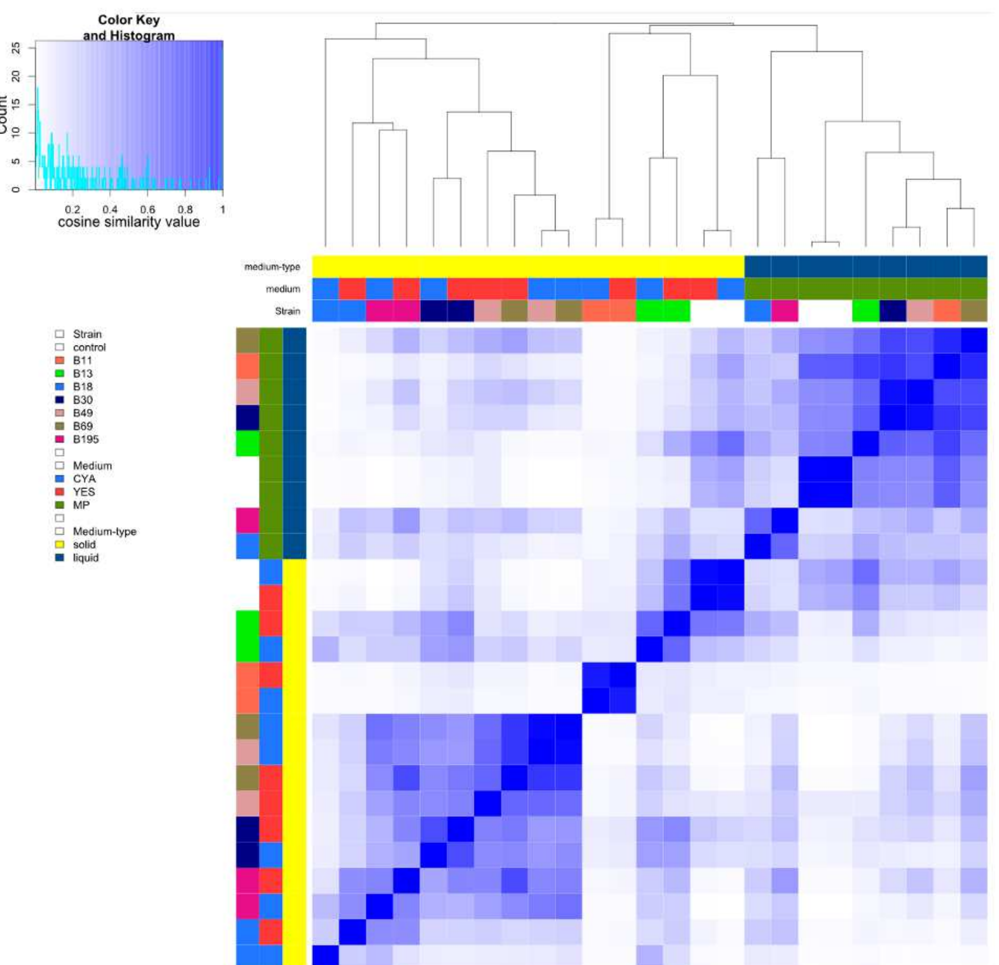

Figure 4. Cosine similarity heat map of extracts analyzed by mass spectrometry.

To support the results of the antimicrobial activity against the bee pathogen *P. alvei*, we expanded the screening panel to include further *Paenibacillus* species as well as the quality-control strain *E. coli* ATCC35218 and type strain *B. subtilis* DSM 10. Interestingly, extract B18 inhibited the growth of *P. lautus* and *P. lactis* at MIC = 0.5 mg/mL (Table 6). The previously observed ability of extract B195 to inhibit *P. alvei* was also observed against *P. lautus* and *P. lactis,* but only at the highest tested concentration (MIC = 2 mg/mL). Extract B195 also displayed moderate activity against *B. subtilis* at 2 mg/mL. None of the extracts inhibited *E. coli* at the concentrations we tested. The full dataset is provided in Supplementary Table S4. Based on the above results, extract B18 was used for micro-fractionation and re-screened against *P. lautus*, for which optimal growth conditions have been established in our laboratory.

The B18 extract yielded five zones of consecutive fractions that inhibited the growth of the test strain, namely fractions 26–28, 30–31, 33–34, 39, and 114 (Table 7). The fractionation, MS, and MS/MS spectra are provided in Supplementary Figures S10–21. Dereplication was achieved by aligning the major ions in active fractions to commercial and in-house MS/MS reference databases. The two main signals in the average mass spectrum of fractions 26–28 were the single protonated ions at m/z 245.1283 $[M+H]^+$ and m/z 211.1438 $[M+H]^+$, corresponding to the molecular formulae $C_{14}H_{16}N_2O_2$ and $C_{11}H_{18}N_2O_2$, respectively. The compounds were identified as the diketopiperazines cyclo-Phenyl- alanyl-Prolyl and cyclo-Leucyl-Prolyl (Figure 5: 1–2). Fractions 30–31 contained a group of stereoisomers of cyclo-Phenylalanyl-Prolyl (Figure 5: 1) as well as m/z 543.2176 $[M+H]^+$, which was assigned the molecular formula $C_{24}H_{34}N_2O_{12}$. Interestingly, this compound did not match any reference compound in our internal MS/MS database, and a molecular formula search in commercial natural product databases (DNP, Dictionary of Natural Products and AntiBase) also retrieved no hits. The identity of the compound remains unknown. An isomer of the same compound was identified in fractions 33–34. In addition, these fractions contained 5,6,8-trihydroxy-3-methyl-2-benzopyran-1-one ($C_{10}H_8O_5$, ionizing at m/z 191.0334 $[M-H_2O+H]^+$, m/z 209.0434 $[M+H]^+$ and m/z 417.0807 $[2M-H_2O+H]^+$) (Figure 5: 3). Fraction 39 contained the siderophore L-ornithine, N^2-acetyl-N^5-hydroxy-N^5-(5-hydroxy-3-methyl-1-oxo-2-pentenyl)-, trimol. ester, (*Z*,*Z*,*Z*)-(9CI) ($C_{39}H_{62}N_6O_{16}$, ionizing at m/z 436.5186 $[M+2H]^{2+}$ and 871.4286 $[M+H]^+$) (Figure 5: 4). A second major ion in fraction 39 (m/z 516.2204 $[M+H]^+$) could not be dereplicated. Ultimately, fraction 114 contained one major ion (m/z 281.2482 $[M+H]^+$) and a minor ion (m/z 379.3368 $[M+H]^+$). Neither compound aligned with any reference compound spectrum. The predicted molecular formula search of the major ion ($C_{18}H_{32}O_2$) retrieved >100 candidates in the DNP, including ubiquitous linolenic acid. However, the nonspecific fragmentation did not allow structural assignment.

Table 7. Summary of the dereplication of active fractions derived from the methanol extract of the *T. purpureogenus* strain B18. The structures are shown in Figure 5.

Fraction	m/z	Adduct	Formula	Name	Structure
26–28	245.1283	$[M+H]^+$	$C_{14}H_{16}N_2O_2$	cyclo-(Phenylalanyl-Prolyl)	1
	211.1438	$[M+H]^+$	$C_{11}H_{18}N_2O_2$	cyclo-(Leucyl-Prolyl)	2
30–31	245.1283	$[M+H]^+$	$C_{14}H_{16}N_2O_2$	cyclo-(Phenylalanyl-Prolyl)	1
	543.2176	$[M+H]^+$	$C_{24}H_{34}N_2O_{12}$	unknown	
33–34	543.2176	$[M+H]^+$	$C_{24}H_{34}N_2O_{12}$	unknown	
	191.0334	$[M-H_2O+H]^+$	$C_{10}H_8O_5$	5,6,8-trihydroxy-3-methyl-2-benzopyran-1-one	3
39	516.2204	$[M+H]^+$	$C_{22}H_{33}N_3O_{11}$	unknown	
	436.5186	$[M+2H]^{2+}$	$C_{39}H_{62}N_6O_{16}$	L-ornithine, N^2-acetyl-N^5-hydroxy-N^5-(5-hydroxy-3-methyl-1-oxo-2-pentenyl)-, trimol. ester, (Z,Z,Z)-(9CI)	4
114	379.3368	$[M+H]^+$	$C_{28}H_{42}$	unassigned	
	281.2482	$[M+H]^+$	$C_{18}H_{32}O_2$	unassigned	

1 2 3 4

Figure 5. Chemical structures of the compounds identified in the active fractions. 1 and 2—diketopiperazines cyclo-(Phenylalanyl-Prolyl) and cyclo-(Leucyl-Prolyl), resp.; 3—5,6,8-trihydroxy-3-methyl-2-benzopyran-1-one; 4—siderophore L-ornithine, N^2-acetyl-N^5-hydroxy-N^5-(5-hydroxy-3-methyl-1-oxo-2-pentenyl)-, trimol. ester, (Z,Z,Z)-(9CI).

4. Discussion

The role of non-pathogenic fungi as regulators of pathogens in the hive and in bee bread has been addressed in several studies [32–34]. Indeed, bee bread is colonized by many filamentous fungi whose potential as biological control agents is yet to be explored. Here, we characterized seven strains of *Talaromyces* recently found in honey bee bread and tested the antimicrobial activity of methanol extracts against bacteria associated with EFB (*P. alvei*) and opportunistic pathogens of the genus *Aspergillus*.

Based on morphology, DNA barcoding, and phylogenetic analysis, we assigned all seven strains to the species *T. purpureogenus* (Samson, Yilmaz, Frisvad, and Seifert 2011), formerly known as *Penicillium purpureogenum* (Stoll, 1904). *Talaromyces* species have previously been recovered from *A. mellifera* honey [80], *A. cerana* bee bread [34], dead adults of *A. dorsata* (Fabricius, 1793) [43], and stingless bees (*Melipona* spp.) [81]. However, to the best of our knowledge, the species *T. purpureogenus* had not been identified in honey bees before, although it has been found in Coleoptera (bark beetles), Diptera (mosquitoes), Hymenoptera (ants), Hemiptera, and Trichoptera [42].

Although few studies have reported the presence of *Talaromyces* in bee bread, its presence is not surprising because this ubiquitous genus can be collected by bees from various sources [54]. Bees passively or actively collect fungi during foraging and incorporate them into the corbicular pollen [82,83]. Interestingly, some fungi are probably introduced or eliminated by the bees during the collection and storage of pollen [30]. We isolated *Talaromyces* strains from some of the hives in one rural location, an urban area, and the city periphery. Seasonal factors may be relevant because we found the *Talaromyces* strains only in spring. Other studies exploring the composition of fungi in bee bread in Europe did not find any *Talaromyces* species, but samples were collected in the summer [29,84], when the absence of *Talaromyces* is consistent with our results. In worker bees, the prevalence of fungi was higher in fall and winter [32]. The composition of fungi in bee bread during months with no flight activity has not been studied.

We did not find any *Talaromyces* species in apiaries located directly on agricultural land. The foraging distance of *A. mellifera* ranges from several hundred meters to several kilometers, depending on the available pasture [85,86]. The presence of agricultural land (such as a canola field) within the foraging range increases the risk of fungicide residues in the food stores that influence the fungal community. The negative effect of agricultural pressure and fungicide contamination on the bee bread fungal community has been confirmed by sequence-based [29] and cultivation-based analysis [87]. Our results suggest that seasonal effects and agricultural pressure determine the presence of *Talaromyces* in bee

bread. However, more research is needed to correlate the presence and seasonal prevalence of *T. purpureogenus* and other *Talaromyces* species in bee bread.

Our in vitro assays revealed that extracts of *Talaromyces* strains B18 and B195 showed antibacterial activity against the pathogen *P. alvei* and other tested strains of *P. lautus* and *P. lactis*. Bioactivity-guided dereplication in assays against *P. lautus* revealed multiple fractions and corresponding inhibitory compounds (siderophores, diketopiperazines, and three unknown compounds) that are also likely to show activity against the other *Paenibacillus* species. Siderophores are metal chelators that facilitate the uptake, intracellular transport, and storage of iron in plants, fungi, and bacteria, making them useful for both medical and environmental applications [88]. Most fungal siderophores are acylated hydroxamates [89], such as the coprogen-type talarazines produced by *Talaromyces* [90]. The biosynthesis of siderophores plays a key role in fungal virulence and influences their interactions with other microbes [91]. The ability to produce iron-chelating molecules can starve some microbes of this essential nutrient and thus cause growth suppression while providing a source of iron for others, such as yeasts, thus promoting their growth [91]. Several fungal compounds have been associated with regulation of the bee bread microbiome, including organic acids [34] and lovastatin [92]. Given that siderophores inhibited the growth of bacteria in our study, it would be interesting to explore the use of siderophores to regulate EFB and AFB (American foulbrood) in vivo. The unknown active compounds should also be investigated as novel anti-infectives.

Most sequence-based studies identify fungi to the genus or species level. Our results highlight the metabolic diversity of different strains of the species *T. purpureogenus*. Depending on the strain and cultivation conditions, *T. purpureogenus* isolated from the bee bread can affect colony health and pathogen resistance in different ways, such as the production of antimicrobial compounds as discussed here, as well as mycotoxins such as the rubratoxins found in our previous study [36]. The genus *Talaromyces* may consist of an assemblage of species/strains with various metabolic activities, as described for bee-related *Aspergillus* species [93]. These fungi are found in the bee hive as a mixture of atoxigenic and toxigenic strains, and some of them are opportunistic pathogens [93]. Similarly, strains of other genera associated with bee bread, such as *Penicillium* and *Alternaria* [94,95], are assumed to be beneficial but can produce both the antimicrobial compounds and mycotoxins that are lethal to bees. Therefore, the nature of the bee bread mycobiome depends on the balance between different fungal strains, which under ideal circumstances are beneficial to the health of bees and the colony as a whole.

5. Conclusions

Fungi in bee bread play an important role in the regulation of pathogens, conferring resistance and promoting colony survival. Many bee bread fungi, including *T. purpureogenus*, can produce protective antimicrobial compounds as well as lethal mycotoxins. The strain-level balance is therefore important for the beneficial function of the mycobiome. Anthropogenic activity, such as the use of fungicides in agriculture, can disrupt this balance and negatively affect colony health.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms11082067/s1, Table S1: Sequences used for phylogenetic analysis. Table S2: *Talaromyces* spp. isolates from *A. mellifera* bee bread collected from different locations in South Bohemia. Table S3: Cosine similarities and grouping of the *T. purpureogenus* strain extracts on different cultivation media. Table S4: Minimum inhibitory concentration (μg/mL) of the crude methanol extracts from *T. purpureogenus* strains. Table S5: Minimum inhibitory concentration (μg/mL) of the reference antibiotics. Figures S1–S5: Phylogenetic trees. Figure S6: Locations of the apiaries, from which the bee bread samples were collected. Figures S7–S9: MS chromatograms of the crude extracts of *T. purpureogenus* strains from different cultivation media. Figures S10–S21: MS and MS/MS spectra of the active fractions and the precursor ions.

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Article

Crude Extracts of *Talaromyces* Strains (Ascomycota) Affect Honey Bee (*Apis mellifera*) Resistance to Chronic Bee Paralysis Virus

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Abstract: Viruses contribute significantly to the global decline of honey bee populations. One way to limit the impact of such viruses is the introduction of natural antiviral compounds from fungi as a component of honey bee diets. Therefore, we examined the effect of crude organic extracts from seven strains of the fungal genus *Talaromyces* in honey bee diets under laboratory conditions. The strains were isolated from bee bread prepared by honey bees infected with chronic bee paralysis virus (CBPV). The antiviral effect of the extracts was also quantified in vitro using mammalian cells as a model system. We found that three extracts (from strains B13, B18 and B30) mitigated CBPV infections and increased the survival rate of bees, whereas other extracts had no effect (B11 and B49) or were independently toxic (B69 and B195). Extract B18 inhibited the replication of feline calicivirus and feline coronavirus (FCoV) in mammalian cells, whereas extracts B18 and B195 reduced the infectivity of FCoV by ~90% and 99%, respectively. Our results show that nonpathogenic fungi (and their products in food stores) offer an underexplored source of compounds that promote disease resistance in honey bees.

Keywords: *Apis mellifera*; antiviral activity; CBPV; fungal extracts; *Talaromyces*; mycotoxins

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1. Introduction

The pollination of plants by insects is a key ecosystem service in agricultural and natural habitats [1]. An estimated 88% of flowering plants are pollinated by animals [2], and 35% of agricultural production is dependent on this process [1]. The recent decline in pollinator activity has therefore raised concerns about the potential impact on biodiversity, the economy and the quality of human life [3,4].

The western honey bee (*Apis mellifera* L., 1758) (Hymenoptera: Apidae) is a generalist pollinator of many plant species [1,4–6]. Multiple factors affect colony health and survival, including the availability of nutrients, beekeeping practices, and the spread of parasites and pathogens [4,7]. Honey bees can be infected by bacteria, fungi, protozoa, and viruses [7–10]. Until recently, viral diseases were considered a low risk, despite occasional outbreaks of infections caused by acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), black queen cell virus (BQCV), and sacbrood virus (SBV). However, the prevalence and virulence of some viruses has increased significantly in the last two decades and is linked to the transfer of the parasitic mite *Varroa destructor* from its original host–the Asian honey bee (*Apis ceranae* Fabricius, 1793)–to *A. mellifera* [11–14]. These mites harm honey bees

by feeding on their hemolymph and fat body, thus impairing their immunity, reproduction and vigor, and, consequently, reducing the likelihood of overwinter survival [14,15]. *V. destructor* is also responsible for the transmission of deformed wing virus (DWV) and viruses representing the so-called AKI complex (ABPV, Kashmir bee virus and Israeli acute paralysis virus) [16–18].

A significant deterioration in bee health has been observed in recent decades, which is most evident in the poor overwintering results recorded in the northern hemisphere. Annual surveys reported average winter losses of 43.7–44.8% in the USA [19–22] and up to 20.9% in Europe from 2012 to 2017 [23–25]. Honey bee losses cannot be attributed to a single cause, but mites and viruses are important factors, and effective control measures are therefore urgently required [26]. Current best practices to control viral diseases in honey bees include introduction of bee stocks with hygienic traits and the management of *V. destructor* infestation using acaricidal treatments [27]. Although control measures against mites are effective, it is not possible to eradicate them completely due to their complex life cycle, which leads to annually recurring problems in beekeeping [27,28]. The control and treatment of viral infections in honey bees has been investigated in many studies [29,30], but it has thus far been impossible to break the cycle of recurring mite infestations and viral infections in order to establish a biological balance [27,31–33].

Natural products are screened for novel compounds against viruses [34] that can also have potential application in beekeeping. Recent studies have shown that some phytochemicals can protect honey bees against parasites and viruses, including caffeine and thymol [35–37]. Furthermore, extracts of some polypore fungi have been shown to inhibit honey bee viruses under laboratory and field conditions [38]. Nonpathogenic filamentous fungi are found naturally in honey bee hives, for example in corbicular pollen and bee bread, and their main function is presumably to preserve the pollen [39–41]. Such commensal fungi have also been shown to antagonize the entomopathogenic fungus *Ascosphaera apis* (Maassen ex Claussen, L.S. Olive and Spiltoir, 1955), which causes chalkbrood disease [42,43], and to regulate the microbial community in stingless bees [44]. However, the role of the fungal community in honey bee hives has not been studied in detail.

We isolated fungi from bee bread and identified several strains of the genus *Talaromyces* (Benjamin, 1955) (Eurotiales: Trichocomaceae). This mold is commonly found in soil, plants, and foods [45]. *Talaromyces* species have previously been identified in honey [46], dead *A. dorsata* (Fabricius, 1793) adults [47], and in the nest and food stores of stingless bees representing the genus *Mellipona* (Illiger, 1806) [48]. In industrial biotechnology, *Talaromyces* species are used to produce enzymes, pigments, and bioactive compounds, including those with antibacterial, antifungal, and antiviral activity [45,49,50]. Given the ability of *Talaromyces* species to produce antiviral compounds [49,51], we decided to test the effect of their crude organic extracts on CBPV infections in *A. mellifera*. CBPV is a taxonomically unclassified, enveloped RNA virus [52] and was identified as the causative agent of chronic bee paralysis in the 1960s [53–55]. Recently, the virus has gained attention due to the rapid increase in the number of cases in some parts of Europe [12]. The symptoms of the disease are easy to identify, and the infection follows classical dose-mortality kinetics under laboratory conditions [53]. CBPV is therefore an ideal model system to test the antiviral activities of fungal extracts in honey bees. Accordingly, we compared the survival of CBPV-infected bees fed on a diet enriched with fungal extracts from *Talaromyces* vs. a control diet. We also tested the antiviral activity of the extracts in mammalian cell culture experiments using model-enveloped and nonenveloped viruses. The analysis of fungal extracts with natural antiviral activity may facilitate the development of biological control methods that improve colony health and fitness and, thus, support agricultural productivity and food security. Such extracts could also be suitable as antiviral therapeutics for human use.

2. Materials and Methods

2.1. Preparation of Fungal Crude Extracts

Fungal strains were isolated from honey bee bread collected in Kamenny Malikov, Czech Republic (49°12′51.533″ N, 15°7′5.129″ E) in March/April 2019. Following their characterization, they were deposited as *Talaromyces* strains in the Fraunhofer strain collection (EXT111748–EXT111754). Seven strains were cultivated in duplicate on Sabouraud dextrose agar (SDA) for 1 week at 25 °C. The mycelia were transferred to a 100 mL malt peptone medium comprised of 30 g/L Difco malt extract (Thermo Fisher Scientific, Hessen, Germany) and 5 g/L mycological peptone from meat (Carl Roth, Karlsruhe, Germany) at pH 5.4 ± 0.2. The cultures were incubated at 28 °C for 6 h, shaking at 175 rpm (ø = 50 mm), and then at 26 °C and 70% relative humidity for a further 18 days as a static culture.

The cultures were freeze-dried and extracted with HPLC-grade methanol (VWR Chemicals, Darmstadt, Germany). Briefly, we added 40 mL of methanol and incubated the samples in an ultrasonic bath for 5–10 min before shaking them for 2 h at 175 rpm (ø = 50 mm). The samples were then passed through filter paper, and the extraction process was repeated. The crude extracts were then dried in preweighed Falcon tubes using a rotary vacuum concentrator (Christ, Frankfurt, Germany), redissolved in 80% acetone, and diluted to a final concentration of 8 mg/mL. The crude extracts and diluted stocks were stored at –20 °C.

2.2. Honey Bee Survival Assay

2.2.1. Honey Bee Feeding and Rearing in vitro

Honey bees were collected from two colonies in the apiary of Justus-Liebig University Giessen, Germany (50°34′06.7″ N 8°40′19.7″ E), between June and August 2021. A piece of a capped brood comb containing late-stage pupae was incubated at 35 °C and 70% relative humidity. Newly emerged bees were collected every 24 h, transferred to conical plastic boxes (18 × 18 × 7.9 cm) and fed on 1:1 (w/v) sucrose solution *ad libitum*. After 3 days, groups of 18–25 bees were housed in conical experimental plastic boxes (11.5 × 8.5 × 8 cm) containing a piece of sterile wax and were fed on the same sucrose solution supplemented with either 40 µg/mL of the fungal extracts or solvent alone (80% acetone) until the end of the experiment. The feeding tubes were weighed and replaced every third day. Feed consumption was recorded as the weight difference before and after tube replacement. A control box identical to the experimental boxes without bees was placed in the incubator to measure the rate of evaporation of the sucrose solution. The average value of the evaporation control was subtracted from the final volume of the solution consumed.

2.2.2. Virus Titration—End Point Replication Assay (BID_{50})

The infectious inoculum was acquired from honey bee pupae infected with CBPV by injection, as previously described [56]. After incubation for 5 days and euthanasia at –20 °C, the bees were placed individually into 2 mL screw-cap tubes containing 830 µL of phosphate-buffered saline (PBS), seven 0.5 mm zircon beads, and two 3 mm glass beads, followed by homogenization (2 × 45 s) in a FastPrep device (MP Biomedicals, Santa Ana, CA, USA). The samples were cleared by centrifugation (18,000 g, 1 min, room temperature), and the supernatants were pooled, aliquoted, and stored at –80 °C.

To determine the infectious loads, the inoculum was titrated and reinjected into honey bee pupae. Capped honey bee brood was cut out of bee combs from healthy bee colonies (specified below) and incubated at 35 °C and 70% relative humidity. Blue-eyed bee pupae (days 13–15 of development) were prepared from the combs and transferred individually to the wells of 24-well plates. Before injection, the virus suspension was thawed on ice, centrifuged (5000 g, 10 min, 4 °C), and a 10-fold dilution series up to $1{:}10^{10}$ was prepared in PBS. Eight pupae were injected in the thorax with 1 µL of each dilution or PBS as a negative control. The pupae were incubated until the time of emergence (day 21 of development). Virus infection was deduced from the death of infected pupae with

developmental stagnation 2 or 3 days after inoculation. The half-maximum bee pupae infectious dose (BID_{50}) of the suspension was calculated using the Spearman and Kärber algorithm (Marco Binder calculator, Department of Infectious Diseases, Molecular Virology, Heidelberg University).

2.2.3. Infection of Adult Honey Bees

After 8 days of feeding with the fungal extracts, the bees were injected with CBPV to assess the effect of the extracts on the infection. To avoid repeated CO_2 exposure, only 3–5 adult bees were anesthetized at a time (within ~10 s) before injection with 1 µL of diluted CBPV into the thorax using a Nanoject II device (Drummond, Birmingham, AL, USA). The living and/or dead bees were counted every day until the mortality reached 100%. Honey bees that died within 24 h post-injection were excluded from the analysis to ensure that only deaths caused by viral infection were counted rather than those caused by handling or wounding.

The experiment was performed in 3–6 replicates and the data were combined. Based on the mortality rates before CBPV injection, 56–124 honey bees were infected for each fungal extract, with the exception of extract B195. The dietary addition of this extract caused high mortality, so only 10 bees were injected in the pilot experiment. Due to the obvious high toxicity of extract B195, this treatment was excluded from subsequent experiments. The data were evaluated in GraphPad Prism 9 using the Kaplan–Meier survival analysis tool.

2.3. Antiviral Activity of the Fungal Extracts in Mammalian Cell-Based Assays

2.3.1. Virus Stock Solutions

An end-point dilution assay was carried out to determine the half-maximum tissue culture infectious dose ($TCID_{50}$) of the nonenveloped feline calicivirus (FCV strain F-9; ATTC VR-782) and the enveloped feline coronavirus (FCoV strain WSU 79-1146; ATTC VR-990) on Crandell-Rees feline kidney (CRFK) cells [57]. The virus suspensions were prepared as a 10-fold dilution series. The diluted viral suspensions (80 µL) were incubated in 96-well plates with 50 µL of the cell suspension (1×10^4 CRFK cells) at 37 °C for 3 days. The $TCID_{50}$ and viral titer were calculated using the Spearman and Kärber algorithm.

2.3.2. Replication/Infection Inhibition Test with Fungal Crude Extracts

We tested four extracts that showed different effects in the bee pupae survival test (B11, B13, B18, and B195). A 10-fold serial dilution of each extract was prepared by mixing 20 µL of the extract with 180 µL of a complete cell culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). The addition of 50 µL of the cell suspension (1×10^4 cells) was followed by incubation at 37 °C for 3 h. We then added 50 µL of the virus suspension (1:20 dilution, corresponding to 1×10^4 $TCID_{50}$ or ~7.5×10^3 plaque-forming units (PFU), and the plate was incubated at 37 °C for 3 days. The number of viral plaques was counted using an inverted cell culture microscope.

2.3.3. Virus Inactivation Test with Fungal Crude Extracts

We mixed 50 µL of the concentrated FCV and FCoV stock (2×10^5 $TCID_{50}$) with the extracts in a 1:10 ratio in 96-well plates. We used 80% acetone as a solvent control. The mixture was incubated for 3 h at 37 °C before preparing a 10-fold dilution series with the pretreated virions. For the measurement of the remaining infectious dose, we added 150 µL of the cell suspension (1×10^4 cells) and incubated at 37 °C for 3 days. Viral titers were determined by the evaluation of cytopathogenic effects.

2.4. Analysis of Fungal Compounds

2.4.1. UHPLC-HR-MS Analysis and Metabolite Annotation

We dried 200 µL of each extract (80 mg/mL) in the rotary vacuum dryer, as described above, then redissolved the residue in 40 µL of methanol and stored it at 4 °C overnight.

The samples were centrifuged (8000 g, 1 min, room temperature) and 30 µL aliquots were transferred to glass vials and processed as previously described [58]. Briefly, the samples were fractionated on a 1290 UHPLC system (Agilent, Santa Clara, CA, USA) equipped with DAD, ELSD, and maXis II (Bruker, San Jose, CA, USA) ESI-qTOF-UHRMS. We used a gradient of 0.1% formic acid in water (buffer A) and 0.1% formic acid in acetonitrile (buffer B) at a flow rate of 600 µL/min. The gradient began at 95% A and was held for 0.30 min before a transition to 4.75% A over 18.00 min and 0% A over 18.10 min, with a hold for 22.50 min. The gradient then increased to 95% A over 22.60 min followed by a hold for 25.00 min. The column oven temperature was set at 45 °C, and the column was an Acquity UPLC BEH C18 1.7 µm (2.1 × 100 mm) with an Acquity UPLC BEH C18 1.7 µm VanGuard Pre-Column (2.1 × 5 mm).

Raw data were processed with DataAnalysis v4.4 (Bruker) using recalibration with sodium formate, followed by RecalculateLinespectra with a threshold of 10,000 and subsequent FindMolecularFeatures (0.5 to 25 min, S/N = 0, minimal compound length = 8 spectra, smoothing width = 2, correlation coefficient threshold = 0.7). Bucketing was performed using ProfileAnalysis v2.3 (Bruker; 30–1080 s, 100–6000 *m/z*, Advanced Bucketing with 24 s 5 ppm, no transformation, Bucketing basis = H1). Bucket annotations were performed using Metaboscape v3.0 (Bruker) based on the HR- *m/z* ratios and isotope patterns. The Bucket annotation list was manually compiled and consisted of ubiquitous *Talaromyces* sp. compounds reported in the literature (Table S1). Fragmentation patterns of the annotated buckets were additionally manually investigated and compared (where available) to those reported in the public GNPS spectral database.

2.4.2. Molecular Networking Analysis

UHPLC-QTOF-MS/MS data were analyzed by molecular networking to allow the variable dereplication of known and unknown metabolites. Firstly, the raw data (.d files) were converted to plain text files (.mgf) containing MS/MS peak lists using MSConvert (ProteoWizard package) [59], wherein each parent ion is represented by a list of fragment *m/z* value pairs (peak picking—vendor MS level = 1–2; threshold—absolute intensity, 1000 most intense). Molecular networking followed established protocols [60] using a cosine similarity cutoff of 0.7. Additionally, ions needed a minimum of six shared fragments (tolerance Δppm 0.05) with at least one partner ion to be included in the final network. In silico fragmentation predictions for a commercial database of compounds [61] (AntiBase 2017) [62] and our in-house reference compound MS/MS database were included in the network as reference substances to narrow down the molecular structure and to highlight compounds of interest. CytoScape v3.4.0 [63] was used to visualize the data as a network consisting of nodes and edges, wherein each node represents a parent ion and its color reflects the sample from which the MS/MS file was obtained. The edge width represents the cosine similarity score between nodes (thick edges indicate high similarity), and the size of the nodes represents the relative abundance of the ion in the extract.

3. Results

3.1. Survival Analysis

3.1.1. Preparation of Extracts and Feeding Assays

Based on ITS sequence analysis, the seven fungal strains were identified as *Talaromyces* with the highest similarity to *T. purpureogenus* (data not shown). Characterization of the strains, including phylogenetic analysis, is still underway, so we cannot yet definitely confirm the species designation. The dried methanol extracts of the seven selected *Talaromyces* strains (Figure 1) resulted in yields of 1.1–5.5 g (Table 1).

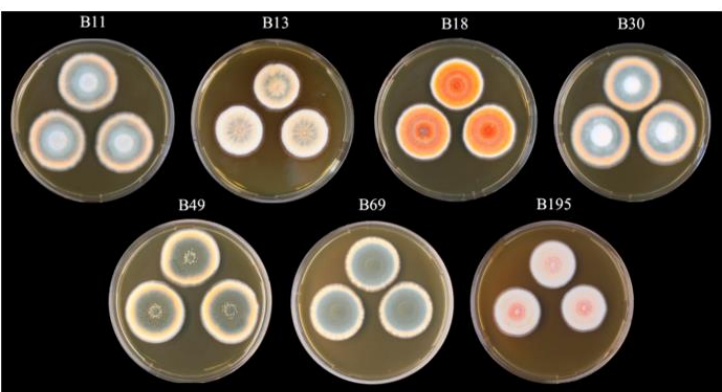

Figure 1. Seven strains of *Talaromyces* used in this study. The cultures were grown on malt extract agar at 25 °C for 1 week.

Table 1. Crude methanol extract yields from the seven *Talaromyces* strains used in this study.

Fungal Strain	Crude Extract Yield [g]
B11	5.52
B13	3.47
B18	1.05
B30	4.42
B49	5.33
B69	3.54
B195	1.24

The average daily consumption of sugar solution containing the fungal extract or acetone as a control was 19.68 mg/bee and did not differ significantly among the groups (one-way ANOVA; $F = 0.63$, $p = 0.741$; Figure 2).

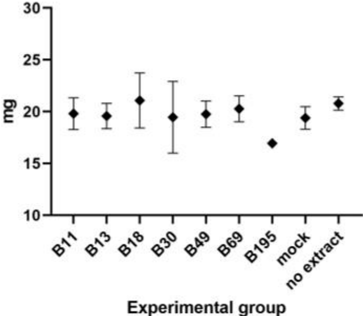

Figure 2. The average daily consumption (mg/bee) of 1:1 (w/v) of sugar solution enriched with the fungal crude extracts, the extraction solvent (80% acetone) in the mock group, or no extract, before the injection of CBPV.

The mortality of the bees after feeding for 8 days reached 1.67–6.67% in most of the groups, but the mortality rate was 50% in the group fed with extract B195. Accordingly, B195 was excluded from further experiments after the first group of 10 bees was tested. In the other experimental groups, 56–124 bees were injected (Table 2).

Table 2. The mortality of bees fed on sugar solution enriched with fungal crude extracts before the CBPV injection or the solvent (80% acetone) in the mock group (followed by mock infection) or no extract in the no extract group (followed by CBPV injection).

Experimental Group	Mortality [%]	No. of Injected Bees
B11	1.67	59
B13	1.67	59
B18	2.36	124
B30	6.67	56
B49	4.00	120
B69	5.00	76
B195	50.00	10
no extract	3.75	77
mock acetone	5.00	76

3.1.2. Determination of an Effective CBPV Infection Dose

The injection of bee pupae with 1 µL of the 10^6-fold CBPV dilution resulted in the death of all inoculated individuals, whereas the 10^7-fold CBPV dilution killed 75% (Figure 3), and the 10^8-fold (or higher) dilution did not differ in effect from the mock infection group injected with PBS. CBPV infection was not detected by RT-PCR in healthy emerging bees representing the 10^7-fold dilution group. The BID_{50}/mL was 3.16×10^{10}, corresponding to a load of ~2.18×10^{10} PFU/mL (calculated using the factor of 0.69). Due to 100% CBPV infection, which resulted in the death of all pupae in the group injected with the 1:10^6 dilution of the CBPV stock, we decided to use this dilution for the evaluation of the fungal extracts.

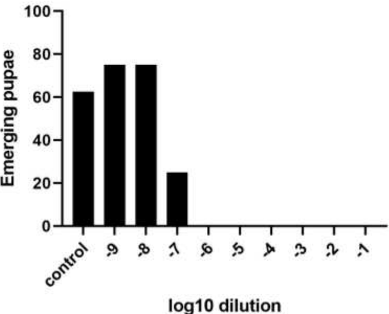

Figure 3. The percentage of surviving (emerging) pupae injected with serial dilutions of the CBPV viral titer and PBS (control).

There were significant differences in the survival curves among the groups (log-rank test; $\chi^2 = 258.80$; $p < 0.0001$). The statistical difference between the survival of the control and CBPV-infected groups (log-rank test; $\chi^2 = 86.14$; $p < 0.0001$) confirmed that the experimental setup was robust. Significant differences compared to the CBPV-infected group were observed in five of the groups fed on the extracts (Figure 4). However, the observed differences in bee survival were positive in only three of the five groups: B13, B18 and B30 (Figure 4B–D).

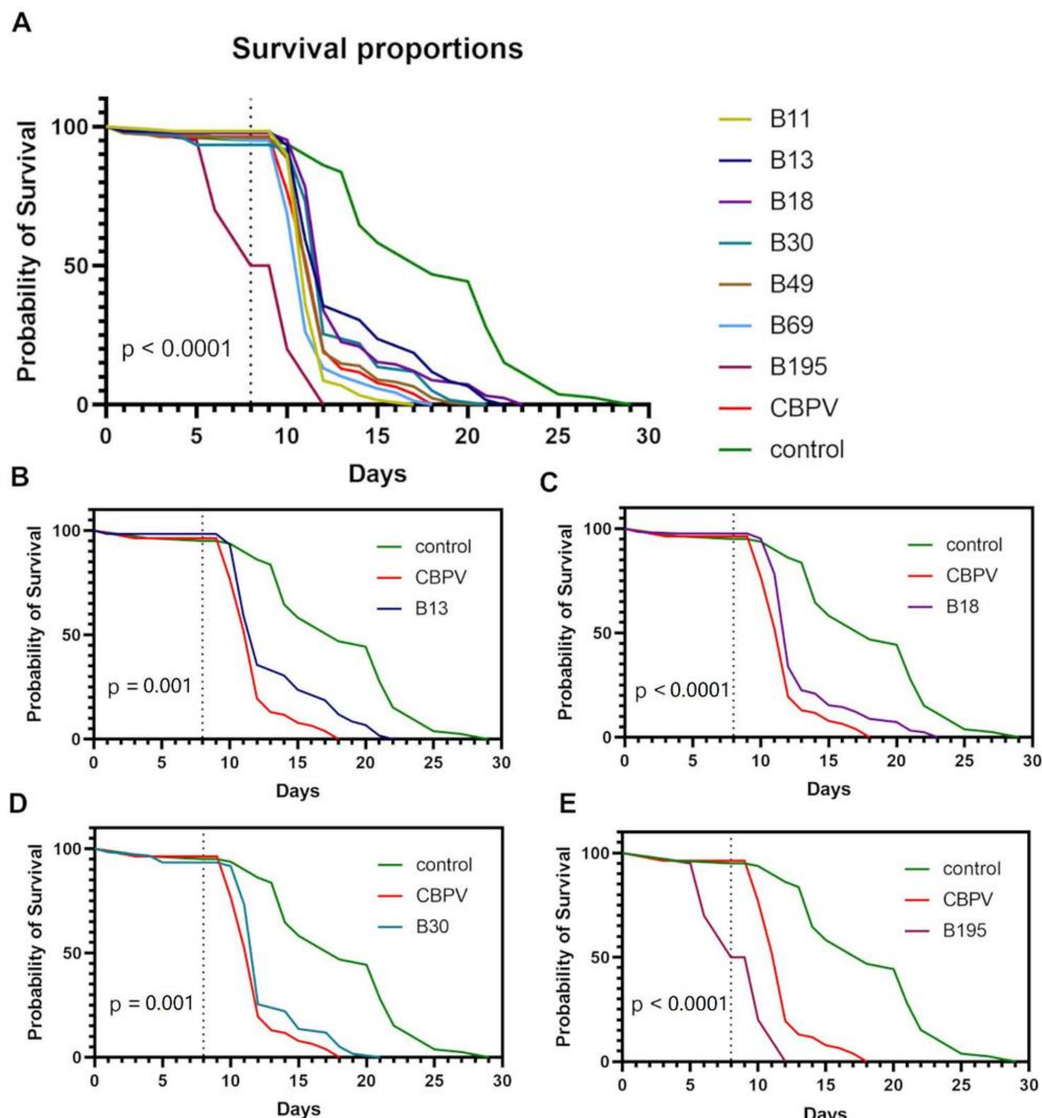

Figure 4. Survival proportions of bees fed on sugar solution containing fungal extracts or solvent (80% acetone) followed by the injection of CBPV or mock infection (control). The dotted line denotes the day of CBPV injection. The top graph shows the results for all seven extracts (**A**). For clarity, the extracts with significant effects are also shown in separate graphs (**B**–**E**), with the exception of B69, which was only just below the significance threshold ($p = 0.044$).

3.2. Antiviral Activity of the Fungal Extracts in Mammalian Cell Culture

3.2.1. Pre-Treated Cells

Virus growth was observed in CRFK cells pretreated with the dilution series of most fungal extracts 3 days after infection with 1×10^4 $TCID_{50}$ of FCV or FCoV as revealed

by the near-complete destruction of the monolayers due to viral lysis. However, in the case of extract B18, we observed a strong and dose-dependent suppression of viral replication, with mostly healthy cells in higher concentrations. The 1:10 dilution of the extract (concentration = 800 μg/mL) was not cytotoxic but strongly inhibited the replication of both viruses and even resulted in the development of an intact monolayer for the cells infected with FCV (Figure 5). Extracts B11 and B195 were highly cytotoxic at a dilution of 800 μg/mL in the noninfected control cells and showed no antiviral protection at higher dilutions. Therefore, any potential antiviral effects of higher concentrations could not be investigated in this assay.

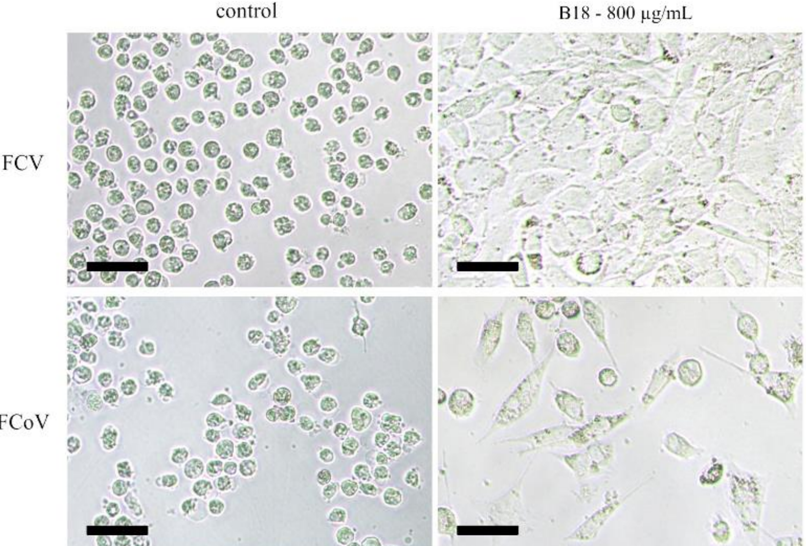

Figure 5. CRFK cells infected with FCV (top left) and FCoV (bottom left) in the control (solvent only) wells. The addition of the B18 extract inhibited the replication of both viruses (right) with a stronger effect on FCV. Note the healthy and infected cells after FCoV infection (bottom right) and the healthy monolayer after FCV infection (top right). Scale bar: 50 μm.

3.2.2. Pretreated Virions

We also incubated the virus suspension with high concentrations of the extracts to investigate their direct virucidal effects. The active ingredients were diluted out during subsequent titration of the virus suspensions, which limited the influence of cellular factors. Contrary to our expectations, the enveloped virus FCoV was unaffected by the solvent-only treatment, which comprised a 1:20 dilution of 80% acetone for 3 h, despite the known mode of action of lipid solvents against viruses [64]. Indeed, the virus titer in the acetone-treated sample was 4×10^6 $TCID_{50}$/mL, whereas 1:20 dilutions of extracts B18 and B195 (400 μg/mL) reduced the virus titer to 1.5×10^5 and 1.5×10^4 focus-forming units (FFU)/mL, respectively (Figure 6). The other extracts had no effect on the FCoV titer. Again, contrary to our expectations, the solvent-only treatment resulted in the complete inactivation of FCV, which is a nonenveloped virus. Due to the unexpectedly strong influence of the solvent, we were unable to evaluate the virucidal effect of the extracts against FCV.

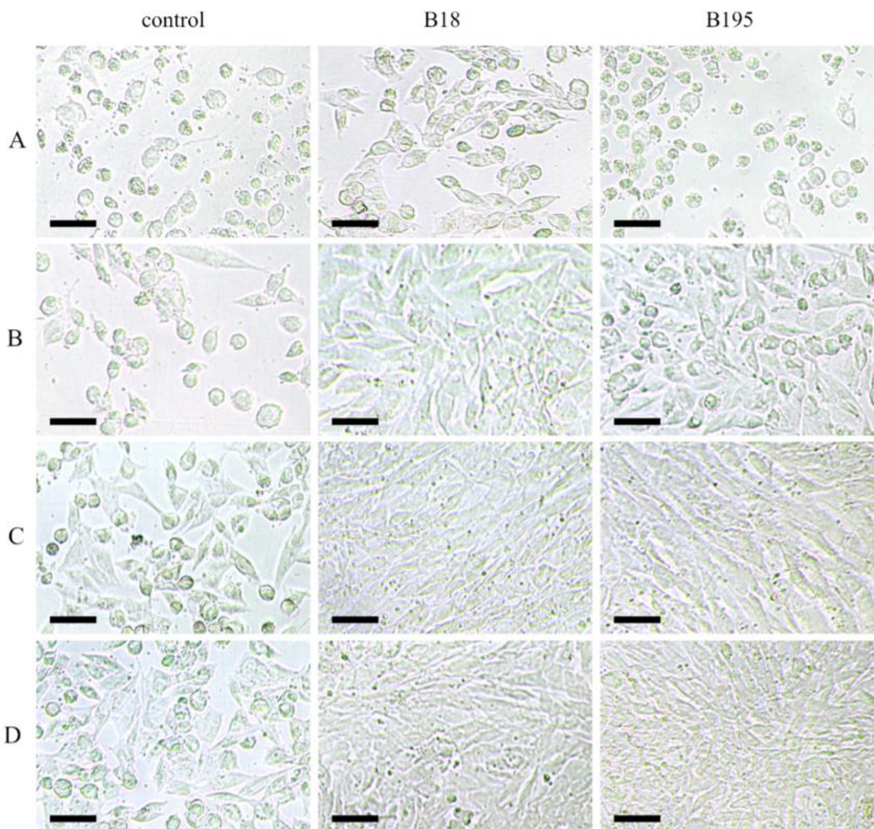

Figure 6. The FCoV stock of 2×10^5 $TCID_{50}$ (control (**A**–**D**)) caused visible symptoms of infection in the CRFK cells, as revealed by the strong cytopathic effects. Pre-incubation of extracts B18 and B195 with the FCoV stock reduced the viral titers by ~99% and ~90%, respectively. Note the cytotoxic effects of the extracts at higher concentrations (B18 and B195, row (**A**)), and the absence of infection in rows (**B**–**D**). Rows (**A**–**D**) represent the 10-fold dilution series of the virus stocks treated with the solvent (control) or extracts B18 and B195. Scale bar: 50 μm.

3.3. Metabolite Annotation

We were able to annotate Rubratoxins A and B, with an annotation confidence level of two as defined by the Chemical Analysis Working Group of the Metabolomics Standards Initiative [65], on the basis of their molecular formulae and identical fragmentation patterns with those deposited in the public GNPS spectral database. The molecular networking analysis revealed the presence of several derivatives (Figure S1). An in-depth analysis of these derivatives was out of the scope of the present report.

4. Discussion

Numerous studies have focused on the biotic and abiotic factors that influence the success of overwintering in honey bees [7,66,67]. However, the colonization of the hive by microbes, including nonpathogenic fungi, has received little attention thus far.

Bee bread contains fungi that are acquired either from honey bee salivary secretions or the collected pollen. Some of these fungal genera are repeatedly found in bee bread [39,40]. However, to the best of our knowledge, this is the first report describing

the genus *Talaromyces* isolated from the bee bread of *A. mellifera*. *Talaromyces* has been found in honey [46], in dead *A. dorsata* (Fabricius, 1793) adults [47], and in stingless bees (*Melipona* spp.) [48]. Multiple studies have reported a relationship between *Talaromyces* spp. and insects from different orders over the last decades [68]. We conducted the first study on the antiviral effects of organic extracts of different *Talaromyces* strains isolated from bee bread.

Our in vivo experiments showed that extracts of *Talaromyces* strains isolated from bee bread had diverse biological effects. The addition of three extracts improved the survival rate and prolonged the average lifespan of CBPV-infected honey bees, but others had no significant impact or were toxic to the bees and therefore increased the mortality rate. Such experiments are complex because they merge direct and indirect effects that are difficult to disentangle, so we complemented the in vivo assay with an in vitro system by testing the fungal extracts in an established mammalian cell culture model. We exposed treated and untreated CRFK cells to the enveloped virus FCoV and the nonenveloped virus FCV. The most potent extracts derived from strains B18 and B195 repressed viral replication in a dose-dependent manner. Although the cells were preincubated with the fungal extracts and only subsequently infected with the viruses, we cannot completely rule out the possibility that the effects were due to virucidal properties of compounds in the extracts. A second virus inactivation assay showed that extract B18 indeed inactivated ~99% of FCoV virions. However, it is likely that additional pharmacological mechanisms were involved in the suppression of viral replication because the virucidal effect alone could not explain the low effective dose of the extract. Several antiviral compounds have been isolated from *Talaromyces* spp., including vanitaracin A, coculnol, and chrodrimanin F [51,69]. The in-depth analysis of compounds in our extracts was beyond the scope of the current study but will be investigated in the future.

The toxic effects of some extracts in honey bees were also confirmed in the cell culture model, where the high cytotoxicity of these extracts prevented the determination of antiviral properties. We assume that such toxic effects are associated with mycotoxins, such as rubratoxins, produced by some *Talaromyces* strains. Recently, rubratoxin B, produced by *T. purpureogenus*, was shown to have insecticidal activity against locusts [70]. Honey bees are exposed to many types of mycotoxins in pollen, such as aflatoxins and ochratoxins, fumosins, zearalenone, and deoxynivalenol, produced mainly by fungi of the genera *Aspergillus*, *Penicillium* and *Fusarium* [71]. Some *Talaromyces* strains used in our study produced rubratoxins A and B (B11, B30, B49, B69 and B195). However, no rubratoxins were found in the extracts of strains B13 and B18, which had a positive effect on honey bee survival. These data suggest that the presence of rubratoxins can increase the mortality of honey bees, although extract B30 (also containing rubratoxins) promoted honey bee survival, so the interactions appear to be more complex. The concentrations of these mycotoxins in the extracts, and the concentrations that are tolerable for honey bees, remain unknown and will be investigated in future experiments.

Optimal conditions for fungal growth and mycotoxin production can deviate significantly. Such differences are also observed among fungal species and between different strains of the same species. Mycotoxin production is often strain-dependent, which is consistent with our results. Furthermore, fungal growth and mycotoxin production depend on many other factors, such as temperature, water availability, pH, and illumination [72,73].

To make pollen storable and to increase the digestibility of its components, honey bees mix collected pollen with nectar and salivary gland secretions and keep it in their wax cells to let it ripen, generating so-called bee bread. The mixture undergoes complex fermentation processes, mainly influenced by lactic acid bacteria and various fungi [39]. The acidic pH, low water activity, high oxidation-reduction potential, and presence of competitive microorganisms create unfavorable conditions for microbial growth, thus protecting bee bread from spoilage [41]. However, some microbes, especially molds and yeasts, can grow slowly in such an environment. Some *Talaromyces* species are particularly tolerant of dry conditions and can grow at a water activity of 0.82, whereas other fungi can grow in a water

activity range of 0.61–0.85 [46]. Furthermore, the presence of nutrients and the absence of competitive bacteria under these conditions can broaden the range of temperatures and water activities suitable for spore germination and growth [72], allowing the production of short (<10 μm) hyphae by a few germinating spores [39]. In the dormant state, the metabolism of fungal spores is shut down to approximately 50% [39,74], but metabolic activity increases greatly during the first phase of germination, where swelling occurs prior to germ tube formation [75].

Our results provide the first description of *Talaromyces* strains in hives of the honey bee (*A. mellifera*) and the relationship between fungal bioactivity and honey bee health under laboratory conditions. By producing antiviral compounds and mycotoxins, *Talaromyces* can protect bees from viruses but can also cause detrimental effects. A more realistic simulation of natural conditions would help us to understand the balance between toxic and antiviral compounds in bee bread colonized by *Talaromyces* and the relevance to honey bee antiviral resistance. This would also be facilitated by the identification of specific compounds that confer the antiviral and toxic effects.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v15020343/s1, Figure S1: Molecular networking analysis; Table S1: List of *Talaromyces* compounds used for the metabolite annotation.

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Article

Culex Y Virus: A Native Virus of Culex Species Characterized In Vivo

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Abstract: Mosquitoes are vectors of various pathogens that cause diseases in humans and animals. To prevent the outbreak of mosquito-borne diseases, it is essential to control vector populations, as treatment or vaccination for mosquito-borne diseases are often unavailable. Insect-specific viruses (ISVs) have previously been described as being potentially helpful against arboviral disease outbreaks. In this study, we present the first in vivo characterization of the ISV Culex Y virus (CYV). CYV was first isolated from free-living *Culex pipiens* mosquitoes in 2010; then, it was found in several mosquito cell lines in a further study in 2018. For mammalian cells, we were able to confirm that CYV does not replicate as it was previously described. Additionally, we found that CYV does not replicate in honey bees or locusts. However, we detected replication in the *Culex pipiens* biotype *molestus*, *Aedes albopictus*, and *Drosophila melanogaster*, thus indicating dipteran specificity. We detected significantly higher mortality in *Culex pipiens* biotype *molestus* males and *Drosophila melanogaster*, but not in *Aedes albopictus* and female *Culex pipiens* biotype *molestus*. CYV could not be transmitted transovarially to offspring, but we detected venereal transmission as well as CYV in mosquitos' saliva, indicating that an oral route of infection would also be possible. CYV's dipteran specificity, transmission routes, and killing effect with respect to *Culex* males may be used as powerful tools with which to destabilize arbovirus vector populations in the future.

Keywords: insect-specific virus; CYV; transmission; host-specificity; fertility

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1. Introduction

Mosquitoes are widespread vectors of various pathogens. In particular, mosquito-borne arboviruses such as dengue virus (DENV), West Nile virus (WNV), Sindbis virus (SINV), and chikungunya virus (CHIKV), to name a few, regularly lead to disease outbreaks in human populations and livestock worldwide [1]. Various factors such as climate change, tourism, and urbanization have led to the spread of mosquito species into new regions and increased incidences of mosquito-borne diseases in recent decades [2]. In addition to invasive mosquito species, resident species can also become vectors for introduced pathogens due to changes in environmental conditions [3]. Since neither effective vaccines nor drug treatment are available against most arboviruses, the common strategy is the prevention of infections by reducing vector populations, for example, through the massive

use of insecticides. However, the use of insecticides bears many disadvantages, including a high risk of insecticide resistance in mosquito populations and harmful effects on the environment and other exposed organisms. Furthermore, repellents are often used to minimize the exposure of susceptible hosts to infected vectors. The disadvantages of repellents, besides the fact that repellents are not 100% effective, include the reduced sensitivity of virus-infected mosquitoes to repellents, which has been described, e.g., for SINV-infected *Aedes aegypti* mosquitoes [4]. Thus, new strategies for reducing arbovirus transmission are needed.

One strategy that has gained increasing attention in the past years is the use of insect-specific viruses or entomopathogenic bacteria and fungi to control vector populations. Insect-specific viruses (ISVs) are viruses from a variety of virus families that can only replicate in insect/arthropod hosts and, therefore, not in vertebrate cells [5]. They have been found in various arthropod species, sometimes due to a specific pathogenicity in their arthropod host, especially in insects cultivated for food or feed [6]. On the other hand, in many cases ISVs have been found incidentally in surveillance studies by screening hematophagous arthropods [5,7,8]. ISVs such as Nhumirim virus (NHUV), Culex flavivirus (CxFV), Palm Creek virus (PCV), Negev virus (NEGV), or cell-fusing agent virus (CFAV) have been described to affect the viral replication and transmission of arboviruses such as WNV [9–11], CHIKV [12], or Zika virus (ZIKV) [13] in vitro and in vivo when co-infected. By potentially affecting the vector's competence or survival and fecundity, ISVs could be a useful tool for vector and virus transmission control. ISVs have also been used as platforms for diagnostic kits or vaccine candidates, e.g., for CHIKV [14]. Mosquito-derived ISVs were found in established cell lines persistently infected, for example, with CFAV, the first described ISV [15], in Aag2 cells (*Aedes aegypti* cells) as well as in laboratory mosquito colonies [10], but also in free-living mosquitoes [16–18]. To understand and exploit the potential of ISVs, more information on the pathogenicity, transmission, and spread of these viruses is needed. In 2010, a member of the *Birnaviridae* family, the Culex Y virus (CYV), was isolated from free-living *Culex (Cx.) pipiens* mosquitoes [17]. The *Birnaviridae* family includes four genera, with three genera (Aquabirnaviruses, Avibirnaviruses, and Blosnaviruses) infecting vertebrates excluding mammals, and a fourth genus, the Entmobirnaviruses, that infects insects [19]. In Germany, the native species *Cx. pipiens* is considered an important vector of WNV [20,21]. This mosquito species forms the so-called *Cx. pipiens* complex, which comprises several biotypes. The biotypes within this complex are morphologically indistinguishable and differ in terms of their essential ecological and behavioral characteristics; however, they can hybridize, thus significantly expanding their host range [22].

To expand our knowledge regarding the interaction of CYV with the *Cx. pipiens* biotype *molestus* mosquitoes, we characterized the virus's replication in vivo, mosquito survival after infection, and the possible transmission routes of CYV. Furthermore, we analyzed the host specificity of this virus by conducting in vitro experiments with mammalian cells and in vivo experiments with the *Cx. pipiens* biotypes *molestus* (mosquitoes), *Aedes (Ae.) albopictus* (mosquitoes), *Drosophila (D.) melanogaster* (fruit flies), *Apis (A.) mellifera* (honey bees), and *Locusta (L.) migratoria* (locusts). All in all, we propose that CYV might be a candidate for new vector control strategies in the future.

2. Materials and Methods

2.1. Insects, Climate Chambers, and Rearing Conditions

The *Cx. pipiens* biotype *molestus* (Forskal, 1775) colony was established in our laboratory in Hannover, Germany, in 2016, and was derived from blood-fed gravid females collected in 2012 in Wendland (W strain), Germany, and from egg rafts collected in 2013 in Langenlehsten (LL strain), Germany [23]. In addition, a hybrid strain from the W and LL strains was established in 2019 and also used for the experiments. The *Cx. pipiens* biotype *molestus* colonies were maintained in climate chambers at 26 °C (±1 °C), a relative humidity of 45–75%, and a 16:8 (light:dark) hr photoperiod with 1 h twilight periods at dusk and dawn. Our *Cx. pipiens* biotype *molestus* mosquito colony was naturally infected with *Wolbachia*

bacteria. They tested negative for Alphaviruses, Orthobunyaviruses and Flaviviruses (data not shown); the primer sets and thermal conditions employed for amplification have already been published [24–27]. An *Ae. albopictus* (Skuse, 1894) colony from Nice, France, was provided by Infravec2 (Laboratory of Prof. Failloux), Nice, France, and was maintained at 28 °C (±1 °C), a relative humidity of 60–75%, and a 16:8 (light:dark) hr photoperiod with 1 h twilight periods at dusk and dawn. Mosquito larvae were reared in plastic basins or bowls filled with dechlorinated tap water and fed with TetraPleco fish food tablets (Tetra Werke, Melle, Germany) until pupation and hatching. Adult mosquitoes were housed in cages (BugDorm-1, 30 cm × 30 cm × 30 cm; Bioquip, Compton, CA, USA) and fed ad libitum with 8% fructose solution. As a dietary supplement, 0.5 g/L of 4-aminobenzoic acid (PABA) was added to the fructose solution. Adult female mosquitoes were fed with dog blood once a week.

Mosquitoes used for the experiments were between two and seven days old. All experiments with mosquitoes were performed in rearing cages (BugDorm-1) in a climate chamber at 26 °C (±1 °C), relative humidity of 45–75%, and a 16:8 (light:dark) hr photoperiod with 1 h twilight periods at dusk and dawn.

Flies of the *D. melanogaster* yellow white (YW) strain from Bloomington Drosophila Stock Center were reared in drosophila cultivation tubes (ø 50 mm; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) filled with approx. 2 cm of corn flour meal agar (35 g of corn flour (Alnatura, Darmstadt, Germany), 8.5 g of dry yeast (Alnatura, Darmstadt, Germany), 23.5 g of glucose (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), 5 g of sugar beet syrup (Bauckhof, Germany), 0.04 g of Nipagin (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), 4 g of agar (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), 1.625 mL of propionic acid (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), and 400 mL of tap water) to provide food and moisture. *D. melanogaster* specimens were held at 24 °C (±1 °C) and 45–55% RH in constant darkness, i.e., without a light:dark cycle.

Locusts were reared in a 12:12 cycle (light:dark) at 30 °C in crowded culture in the facilities of the Institute of Physiology and Cell Biology of the University of Veterinary Medicine Hannover. The animals were fed ad libitum with fresh wheatgrass and wheat bran. Adult locusts were offered tubes filled with moist sand and water-soaked vermiculite for egg laying. Egg tubes were incubated at 30 °C until eclosion.

Honey bees were collected from two *A. mellifera* colonies from the apiary of the Justus-Liebig University (Giessen, Germany, 50°34′06.72″ N 8°40′19.7″ E) in the second half of August 2021. A piece of capped brood comb with late-stage pupae was incubated at 35 °C and 70% relative humidity. The newly emerged bees were transferred to a conical plastic box (18 cm × 18 cm × 7.9 cm) and fed sugar solution (1:1 sucrose and water) ad libitum.

2.2. Cell Culture

Mammalian cells used for infection experiments were BHK-21 (hamster kidney cells; CCVL L 0179) and A549 (human lung epithelial cells; ATCC® CCL-185™). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Thermo Scientific Inc., Waltham, MA, USA) (A549) or Minimum Essential Medium (MEM; Capricorn Scientific, Ebsdorfergrund, Germany) (BHK-21), both supplemented with (1%) penicillin–streptomycin (100 units/mL) (PAN-Biotech GmbH, Aidenbach, Germany), (1%) L-glutamine (200 mM) (PAN-Biotech GmbH, Aidenbach, Germany), and 10% FBS (A549) or 5% FBS (BHK-21) (Capricorn Scientific, Ebsdorfergrund, Germany) at 37 °C and 5% CO_2. Cell cultures were split twice weekly to prevent cells from reaching full confluence. To detach the cells from the bottom of the flask, cells were treated with trypsin.

2.3. Homogenization, RNA Extraction, RT-PCR, qRT-PCR, and CYV Standard Preparation

All insect samples were homogenized before further analyses. For this purpose, insect samples were transferred in groups or individually, depending on the assay, to reaction tubes containing 500 µL of Schneider's Drosophila Medium (PAN-Biotech GmbH, Aidenbach, Germany) or 1 mL of nuclease-free water (bees) containing two 5 mm steel

balls (Isometall, Pleidelsheim, Germany) and were homogenized using the TissueLyser II (Qiagen, Hilden, Germany) at 30 Hz for 30 s or 25 Hz for 3 min (bees). Samples were then centrifuged at full speed (13,300× *g*) for 1 min, and the homogenate was frozen at −18 °C until further processing. RNA extraction was performed using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

To quantify CYV RNA copy numbers, primers OSM_318 (5′ CCAGAGAATGTGAAGAGA 3′), OSM_319 (5′ CGTTGTTAAGGAAGACTC 3′), and a probe OSM_320 (FAM-CACAAGAGGATACACAAGCAGCG-BHQ1) were used. The Luna Probe One-Step RT-qPCR Kit (New England Biolabs, Ipswich, MA, USA), was used for all samples with 9 μL master mix and 1 μL template in an AriaMx Real-time PCR System (Agilent Technologies, Santa Clara, CA, USA). The thermal program consisted of the application of 55 °C for 10 min, followed by 1 min at 95 °C and 40 cycles of 10 s at 95 °C and 30 s at 54 °C (annealing). The resulting fragment was 114 bp long.

A PCR standard was prepared using the SuperScript III One-Step RT-PCR Platinum Taq DNA Polymerase Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions, thus reducing the final volume from 50 μL to 25 μL, including 1 μL template. A fragment length of 414 bp was amplified using primers OSM_321 (5′ GGCAATGTTCGGGCTCATC 3′) and OSM_322 (5′ CGACAGTGCAGGAGTAGGGG 3′). The cycler program was set to 1 min at 60 °C and 45 min at 50 °C, followed by 2 min 95 °C for reverse transcriptase. This was followed by 40 cycles of DNA denaturation at 94 °C (15 s), annealing at 59 °C (30 s), and elongation at 68 °C (30 s). A final elongation of 7 min was carried out. The resulting PCR product was then purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey and Nagel, Düren, Germany) according to their instructions. For transcription, a T7 promoter was added to the previous purified PCR product. For this purpose, a T7 promoter was added to the standard OSM_321 forward primer, OSB_728 (5′ TAATACGACTCACTATAGGGGGCAATGTTC 3′). The RNA concentration was measured, and the RNA copy number/mL was calculated. The detection limit for our standard was 1×10^4 RNA copies, which had Ct values between 33 and 35; thus, measurements over Ct value 35 were excluded in further analyses.

2.4. Injection

CO_2-anesthetized adult mosquitoes were injected in the lateral metathorax [28,29] using a Nanoject II Auto-Nanoliter injector (Drummond Scientific Company, Broomall, PA, USA). The injection solution used was either the CYV isolate of Franzke et al. (2018) [30] (first isolated in 2010 by Marklewitz et al. [17]) (virus-infected groups) or Schneider's Drosophila Medium (control groups). The injection rate was set at 23 nL per second. All experiments were performed in at least three individual replicates; bees' survival was replicated in two independent replicates. An RNA standard was used to quantify CYV because the virus did not show a cytopathic effect in any of the cell cultures tested. Therefore, we used the RNA copy numbers to standardize our experiments and ensure reproducibility. Mosquitoes were then injected with 46 nL.

2.5. In Vivo Growth Kinetics in Cx. pipiens Biotype Molestus

In vivo kinetics of viral growth in *Cx. pipiens* biotype *molestus* were monitored by measuring viral RNA copy numbers at the following days post-injection: zero, three, seven, and fourteen. Mosquitoes from all three described strains (W, LL, and hybrid) were sampled in equal numbers of males and females. To study viral growth and dissemination, mosquitoes were injected with 46 nL of CYV (approximately 3.32×10^5 RNA copies) and RNA copies were measured by qRT-PCR using the CYV standard described in Section 2.3. In total, 72 males and 72 females were dissected starting on the day of injection, wherein mosquitoes were dissected within two hours after injection. On each dissection day, six males and six females (two individuals per sex per mosquito strain) were anesthetized with CO_2 and immobilized by removing legs and wings. The immobilized mosquitoes were then rinsed with 70% ethanol to sterilize their exoskeletons and remove static charge from the mosquitoes;

then, they were placed on a microscope slide in 50 µL of 1× phosphate-buffered saline (PBS; pH 7), which provides necessary physiological conditions and prevents contamination of the hemolymph. The salivary glands (females only), ovaries or testes, intestines, heads, and carcasses were dissected and washed three times in 200 µL 1× PBS and then pooled in 1.5 mL reaction tubes containing 500 µL Schneider's Drosophila Medium and two 3 mm steel balls and stored at −18 °C until further processing.

2.6. Transmission of CYV within Cx. pipiens Biotype Molestus Populations

2.6.1. Vertical Transmission

To test vertical transmission of CYV within mosquito populations, a total of 126 virgin males and 324 virgin females were infected with CYV by injection and a control group, containing 108 virgin females and 42 virgin males, was injected with Schneider's Drosophila Medium. To ensure virginity on the day of injection and prior to assembly, pupae were placed in individual *Drosophila* tubes until hatching. After injection, males and females were placed in BugDorm-1 cages and fed ad libitum with an 8% fructose solution under conditions described in Section 2.1. The progeny (adults of the F1 generation) from autogenous oviposition of CYV-infected mosquitoes were reared and tested for CYV.

2.6.2. Venereal Transmission

Virgin mosquitoes were injected and then incubated again separately sexed for five days. On the fifth day, CYV-infected mosquitoes were joined with virgin CYV-naïve mosquitoes of the opposite sex. After seven days, the surviving individuals, consisting of a total of 168 CYV-injected females, 127 CYV-injected males, 70 CYV-naïve females, and 71 CYV-naïve males, were pooled into a maximum of twelve individuals per pool and sexed and processed as described for qRT-PCR analyses. The minimum infection rate (MIR) was calculated for this experiment as described by Pettersson et al. (2014) [31], since all samples were tested in pools. The number of individuals in each pool was different; thus, RNA copy number per animal was calculated for statistical analyses.

2.6.3. Saliva Assay

For the oral transmission experiments *Cx. pipiens* biotype *molestus* mosquitoes were injected with CYV two to three days after hatching, as described earlier, and caged until five days post-infection (dpi) to ensure viral growth. We examined saliva from 30 CYV-injected males and 30 CYV-injected females. Saliva was obtained by forced salivation of male and female mosquitoes via removal of wings and legs and placing the proboscises in 10 µL of 1× PBS using a pipette tip. Mosquitoes were allowed to feed for 1.5 h. Saliva and mosquito bodies were then tested for CYV by qRT-PCR.

2.6.4. Oral Infection

Furthermore, we performed viral growth kinetic experiments on orally CYV-infected mosquitoes with a total number of 144 CYV-infected mosquitoes comprising 72 males and 72 females. An 8% fructose solution containing 35 µL of red ink as a dye and 5 µL of CYV containing approximately 3×10^6 RNA copies was prepared. Feeding was performed in tubes in groups containing a maximum of 12 animals each. The mosquitoes were allowed to feed on the fructose solution containing CYV via cotton swabs (200 µL per cotton swab) for 180 min. Animals with distinctly red-colored abdomens were used for growth kinetics experiments, as described in Section 2.5.

2.7. Oviposition and Progeny Outcome of CYV-Injected Cx. pipiens Biotype Molestus

All autogenous egg rafts laid by CYV-injected females described in Section 2.6.1 were photographed using a Moticam 5 camera (Motic, Barcelona, Spain) and the Motic Images Plus 2.0 software (Motic, Barcelona, Spain), and the quantities of egg rafts and eggs were counted using ImageJ 1.53c software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Then, the egg rafts were kept in tanks filled with tap water,

and hatched larvae were fed 70–80 mg of a TetraPleco fish-food tablet daily. From the late L_2 instar to the early L_3 instar, 100 larvae were kept at a constant density of 4 cm^3/larvae to prevent the crowding factor from affecting development. Resulting pupae were counted and hatched mosquitoes were frozen at −18 °C.

2.7.1. F1 Generation—Progeny of CYV-Infected Females of *Cx. pipiens* Biotype *Molestus*

Wings from 295 frozen female offspring and 286 frozen male offspring from CYV-infected mosquitoes and from 102 female and 97 male offspring from medium-infected mosquitoes were severed near the thorax with precision forceps and wing length (WL) and wing area (WA) were measured. The detached wings were placed on a white sheet of paper with a scalable grid for size reference. A glass slide was carefully placed on the wings to straighten them. Wings were photographed using a Moticam 2.0 camera on a binocular microscope at 10× magnification and measured using ImageJ 1.53c. Wing length was measured using the linear distance between the distal end of the alula and the tip of the third radial vein at the apical margin. Wing area was defined as the area on the upper surface excluding the fringe. The proximal end of the area was defined by a vertical line connecting the end of the fringe and the posterior margin of the alula [32]. WL and WA were measured for the left and right wings and the final WL was determined as the mean of the size of the right (WLR) and left (WLL) wings [(WLR + WLL)/2]. The same was performed for the WA [(WAR + WAL)/2].

2.7.2. Survival Assays

We monitored the survival of 96 CYV-injected *Cx. pipiens* biotype *molestus* males and 96 CYV-injected *Cx. pipiens* biotype *molestus* females. A control group injected with Schneider's Drosophila Medium that comprised the same number of mosquitoes was also included in each replicate. Mosquitoes were injected as described in Section 2.4, and observation began 24 h post-injection to exclude dead mosquitoes due to injection. We then monitored mosquito survival daily over a 14-day period and removed any dead individuals from the cages. At least 10% of dead individuals were tested for CYV to validate infection status.

2.8. In Vitro CYV Infection of Mammalian Cell Cultures

Mammalian cells (BHK-21 and A549) were seeded in 12-well plates (Sarstedt AG & Co. KG, Nümbrecht, Germany) at 0.1×10^6 cells per well with 500 µL of the appropriate cell culture medium and 10% FBS and allowed to grow to approximately 80% confluence. Approximately 3×10^7 CYV RNA copies were added to 500 µL of Schneider's Drosophila Medium and used for infection. Mammalian cells were inoculated at 37 °C and 5% CO_2 for one hour, with plates gently shaken every 15 min. As stated above (2.4), CYV did not induce any CPE in cells, thus hindering a regular plaque-forming unit assay; therefore, we used RNA copies of the virus to standardize our experiments. After inoculation, the inoculum was removed, and the cells were washed three times with 500 µL 1× PBS (pH 7) and replenished with medium and FBS. Viral kinetics were monitored for seven days, with 200 µL of supernatant collected on day zero, day three, and day seven post-inoculation. Infection and negative controls were performed and treated in triplicate and in three independent experiments.

In Vivo CYV Infection of Other Insects

We further investigated the host specificity of CYV by injecting the virus into other insect species. For all infection experiments, each replicate included a virus-infected group and a Schneider's Drosophila Medium control group. Individuals that died within 24 h post-injection were excluded from further analyses.

For this investigation, *Ae. albopictus* mosquitoes were injected with 46 nL of CYV (approximately 3.32×10^5 RNA copies), as described in Section 2.4. A total of 144 mosquitoes consisting of 72 males and 72 females injected with CYV and the corresponding control groups were observed in survival experiments conducted as described in Section 2.7.2.

Dead individuals were removed and tested for CYV via qRT-PCR. Viral growth was tested in pools of five males and five females per pool after CYV infection at days zero, one, seven, ten, fourteen, and twenty-one via qRT-PCR, and viral growth was determined.

D. melanogaster flies of a yellow white wildtype (YW) strain, as representatives of Brachycera, were injected with 18.4 nL of CYV (approximately 7.34×10^3 RNA copies) or Drosophila Schneider's Medium as control group, respectively. In total, 125 males and 125 females per virus and control group (three to six days old) were observed. For the experiments, two to three *D. melanogaster* tubes (ø 50 mm; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) of strain YW were completely emptied. On the third day after hatching, the newly hatched individuals were placed on fresh drosophila cultivation tubes (ø 50 mm; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) filled with approx. 2 cm of corn meal agar and left for three days at 24 °C (±1 °C) and 45–55% RH in constant darkness to allow the immune system of flies fully develop. After injection, 50 flies (25× males and 25× females) per group were placed in fresh *Drosophila* tubes containing cornmeal agar and maintained under the same conditions as *Cx. pipiens* biotype *molestus* mosquitoes. The flies were checked every day for survival, except on weekends. Dead flies were removed from the tubes and stored at −18 °C. After seven days post-infection, the flies were replaced in new *Drosophila* cultivation tubes to ensure that generations did not mix. We performed survival experiments over 14 days. Dead individuals were removed from the tubes and tested for CYV via qRT-PCR. For viral growth, fruit flies were injected as described and individual pools of five males and five females per pool were tested for CYV at day zero, one, five, ten, and fourteen after injection.

Specimens of *A. mellifera*, the honey bee, a member of the Hymenoptera order, were injected with 1 µL of CYV (approximately 8.17×10^5 RNA copies) or Schneider's Drosophila Medium. The bees were quickly anesthetized with CO_2 and their thoraxes were injected using Nanoject II (Drummond Scientific Company, PA, USA). Subsequently, the bees were distributed into the plastic experimental boxes (11.5 cm × 8.5 cm × 8 cm) with a piece of wax, provided with sucrose solution (1:1) ad libitum, and kept under the conditions described above. A total of 29 bees were injected with CYV and 51 were injected with medium for survival experiments described in Section 2.7.2.

For the study of viral growth kinetics, 36 bees were injected as described above and samples were analyzed for CYV by qRT-PCR at four time points post-injection: zero, three, seven, and fourteen. Two live bees per treatment were frozen and stored at −20 °C until further analysis.

L. migratoria, a member of the order Orthoptera, was also injected with CYV. For each treatment group (Schneider's Drosophila Medium as control and the CYV-injected group), 24 first-instar locusts were injected a few hours after eclosion [33] with a volume of 193.2 nL (3 × 64.4 nL) of either Drosophila Schneider's Medium as control or CYV (approximately 4.98×10^5 RNA copies). Locusts were kept in groups of six in *Drosophila* tubes filled up to 2 cm with agar to provide adequate humidity and were maintained at ambient conditions in the laboratory (temperature: 22–23 °C; humidity: 20–40%). The agar-filled tubes were replaced every second day, and the animals were fed with fresh or thawed wheatgrass daily. We performed survival experiments as described in Section 2.7.2, and dead individuals were tested for CYV via qRT-PCR.

2.9. Statistical Analyses

All statistical analyses were performed using Prism 9 Version 9.4.0 (San Diego, CA, USA). To determine the survival rates, Kaplan–Meier survival curves were generated and significance levels between curves were determined using the log-rank test.

Data were tested for normality using the Shapiro–Wilk test. Parametric analyses of more than two groups were conducted with a one-way ANOVA test or two-way ANOVA test, while non-parametric data were tested using the Kruskal–Wallis test. Individual testing of two groups was performed using the unpaired *t*-test with Welch's correction for parametric data and the Mann–Whitney test for non-parametric data. Multiple *t*-tests were

performed using Bonferroni correction. Results of tests were considered significant when $p \leq 0.05$. Significance levels were displayed in four levels by different numbers of asterisks: $p \leq 0.05$ is shown as *, $p \leq 0.01$ is marked with **, $p \leq 0.001$ with ***, and $p \leq 0.0001$ is marked with ****.

3. Results

3.1. In Vivo Growth Kinetics in *Cx. pipiens* Biotype Molestus

To investigate viral replication and dissemination in vivo, the viral growth kinetics of CYV were examined. Accordingly, viral RNA was measured in the heads, carcasses, reproductive organs, intestines, and salivary glands (females only) at four time points after CYV injection (zero, three, seven, and fourteen dpi) in *Cx. pipiens* biotype *molestus* mosquitoes. In total, we dissected 72 males and 72 females divided into 3 independent replicates. The results show that within two hours after its injection into the thorax, all organs were flooded with CYV, although all organs were washed three times in 1× PBS. We obtained an increase in RNA copy numbers in all the tested organs of the females at day three and day seven post-injection (Figure 1A). Except for the ovaries and carcass, where a slight decrease in RNA copy numbers was observed, the RNA increase could still be measured on the fourteenth day post-injection. The kinetics of viral growth in the males after injection showed a similar pattern as that in the females, with an additional slight decrease in the intestine on the fourteenth day (Figure 1B). In almost all the analyzed samples, the number of RNA copies was slightly lower in the males compared to the females but did not differ significantly (for all organs, $\alpha > 0.05$; Welch's *t*-test) (Figure 1A,B). The lowest RNA copy number was measured in the intestine of the males with 7.15×10^2 RNA copies on the day of injection. The highest RNA copy numbers were measured in the carcasses of both sexes at all sampling days, ranging from 3.28×10^4 at day zero to 1.08×10^7 at day fourteen in females and 3.70×10^4 at day zero to 8.91×10^6 at day fourteen in males. The lowest RNA copy numbers were found in the females' salivary glands and the intestines of males and females on the day of injection. The mean RNA copy numbers of all the organs combined yielded a copy number of 4.44×10^4 in males and 4.56×10^4 in females on the day of injection. Compared with the RNA copies in the inoculum (3.32×10^5 per injection), the measured RNA copies in the mosquitoes' organs were lower after injection. On the fourteenth day, higher amounts of viral RNA were measured for all organs compared to the inoculum, namely, 1.16×10^7 in males and 1.40×10^7 in females. The data shown include measurements that passed the detection limit.

Statistical analysis of viral RNA copy numbers per organ and sex over four time points showed that, with the exception of the carcass, no significant differences in viral growth were detected ($\alpha > 0.05$; Multiple *t*-test, Bonferroni-corrected). In the carcasses, significantly lower viral RNA copy numbers were detected in both sexes at day zero compared to day seven (males: $p = 0.0177$; Multiple *t*-test, Bonferroni-corrected; females: $p = 0.0425$; Multiple *t*-test, Bonferroni-corrected) and day fourteen in males ($p = 0.0086$; Multiple *t*-test, Bonferroni-corrected). In addition, a significant difference was observed between day zero and day three in males ($p = 0.0141$; Multiple *t*-test, Bonferroni-corrected) (Table 1).

Table 1. Summary of statistical analysis of viral growth in male and female carcasses. Multiple *t*-tests with Bonferroni correction were performed to determine *p*-values; below, the *p*-values are shown for carcasses of both sexes compared between all measured time points tested.

	Carcass					
	0 vs. 3 dpi	**0 vs. 7 dpi**	**0 vs. 14 dpi**	**3 vs. 7 dpi**	**3 vs. 14 dpi**	**7 vs. 14 dpi**
Males	0.0141	0.0177	0.0086	ns	ns	ns
Females	ns	0.0425	ns	ns	ns	ns

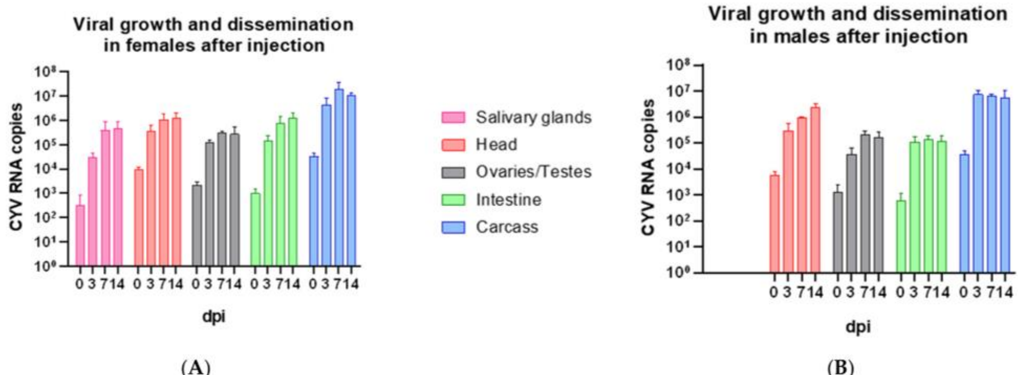

Figure 1. Growth kinetics of CYV after injection in *Cx. pipiens* biotype *molestus*. Mosquito dissection and preparation of different organs at zero dpi, three dpi, seven dpi, and fourteen dpi. Mean RNA copy numbers per organ per individual are displayed per dissection day with SD. (**A**) Viral growth in organs of 72 females; (**B**) viral growth in organs of 72 males. Pink—salivary glands; red—head; grey—ovaries/testes; green—intestine; blue—carcass; dpi = days post-infection.

3.2. *CYV Transmission Experiments with Cx. pipiens Biotype Molestus*

We investigated how CYV could be transmitted within a mosquito population. For this purpose, we tested different transmission routes in *Cx. pipiens* biotype *molestus* mosquitoes, namely, vertical, venereal, and oral transmission, in the following set of experiments.

3.2.1. Vertical Transmission

To investigate whether the virus could be transmitted vertically, we first examined CYV replication in the reproductive organs of both sexes (Figure 1A,B). We found that CYV replicated in the reproductive organs of the males and females at nearly identical levels, ranging from 1.35×10^3 and 2.16×10^3 RNA copies in the testes and ovaries, respectively, on day zero to 1.67×10^5 and 2.72×10^5 on day fourteen. The peak viral RNA was measured on day seven in both sexes, with 3.03×10^5 in the ovaries and 2.06×10^5 in the testes.

Next, we tested the vertical transmission of the virus in the CYV-positive mosquitoes to their offspring. Therefore, 672 adult F1 generation mosquitoes were tested for viral RNA copies; consequently, it was revealed that none of the tested samples had tested positive. Therefore, it seems that the vertical transmission of CYV does not occur in *Cx. pipiens* biotype *molestus* mosquitoes.

3.2.2. Venereal Transmission

Another potential route of viral spread within a population might be the venereal transmission of CYV from CYV-positive to CYV-naïve mosquitoes. The RNA copy numbers were analyzed per mosquito pool; in a second step, we calculated the RNA copy number per individual animal from these data to enable better comparability between pools (Figure 2). To test this, we mated CYV-positive males or females with their non-infected counterparts. We were able to detect CYV RNA in all the tested pools of the priorly CYV-naïve females and males. The samples were tested in pools, which resulted in a minimum infection rate (MIR) of 8.57% for the females (six pools) and 9.86% for the males (seven pools). The transmission occurred both from females to males and from males to females. We measured a mean RNA copy number of 1.09×10^7 12 days post-injection for the females, which was calculated per animal. Their male mating partners, who were originally CYV-naïve in this experiment, had a mean RNA copy number of 3.97×10^3 per animal. Similar results were observed for the CYV-injected males 12 days after injection, with a mean RNA copy

number per animal of 1.21×10^7. An average of 2.65×10^3 viral RNA copies were detected in their female, previously CYV-naïve mating partners.

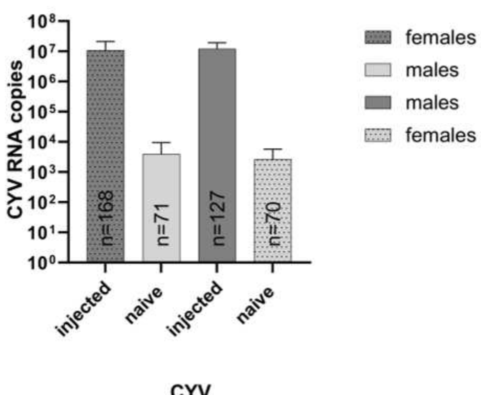

Figure 2. CYV measurement of previously CYV-naïve *Cx. pipiens* biotype *molestus* mosquitoes after mating with CYV-positive mating partners. Shown are mean viral RNA copies measured by qRT-PCR calculated for one mosquito (per pool) seven days post-mating, displayed with SD. Dark grey with dots: CYV-injected females (n = 168); dark grey—CYV-injected males (n = 127); light grey with dots: CYV-naïve females (n = 70); light grey—CYV-naïve males (n = 71).

3.2.3. Oral Transmission

To investigate whether CYV could be transmitted orally in the mosquito populations, first, we conducted a forced salivation assay with the CYV-injected mosquitoes and additionally performed feeding experiments to determine whether the virus could be ingested in the diet and replicate in the mosquitoes. Forced saliva testing confirmed that 100% of the CYV-injected mosquitoes were positive for CYV infection after injection. Regarding the females, 86.7% also had CYV-positive saliva, with a mean RNA copy number of 7.76×10^3, while 63.3% of the males had CYV-positive saliva with a mean RNA copy number of 8.62×10^3 in the male saliva (Figure 3). RNA copy numbers in the bodies of females and males were significantly higher (for both sexes: $\alpha < 0.0001$; Mann–Whitney test) than in their respective saliva samples: 2.14×10^7 (females) and 3.69×10^7 (males).

In a second step, we found that CYV can be taken up via a fructose solution and that the virus replicates after oral uptake in both sexes (Figure 4). All organ pools of fed males and 66.7% of the female pools were tested positive for CYV RNA after feeding. The RNA copies of CYV decreased in all organs of both sexes from the day of infection to day three post-infection, indicating the digestion of the initial inoculum. No virus was detected in the reproductive organs and intestines of the females and males on the third day after oral uptake. However, after seven days of infection, an increase in RNA copies was observed in all the organs except the female salivary glands and the head, in which no viral RNA was detected. Fourteen days after oral infection, no viral RNA copies were detected in the females' carcasses and the highest in the males' carcasses with 4.29×10^4 RNA copies. The mean RNA copy numbers of all organs combined resulted in a copy number of 4.98×10^3 in the males and 7.07×10^3 in the females on the day of oral infection.

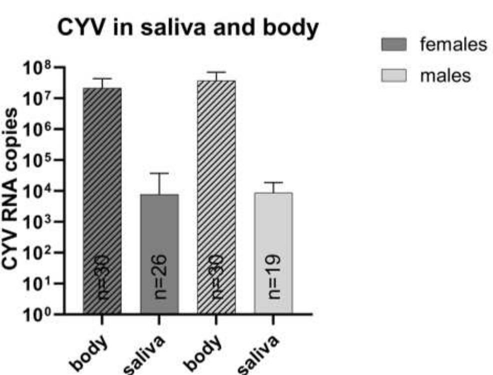

Figure 3. CYV measurement in saliva and carcasses five days after injection in *Cx. pipiens* biotype *molestus* mosquitoes after forced salivation. Shown are mean viral RNA copies measured by qRT-PCR per mosquito displayed with SD. Dark grey with lines—female bodies (n = 30); dark grey—female saliva (n = 26); light grey with lines—male bodies (n = 30); light grey—male saliva (n = 19).

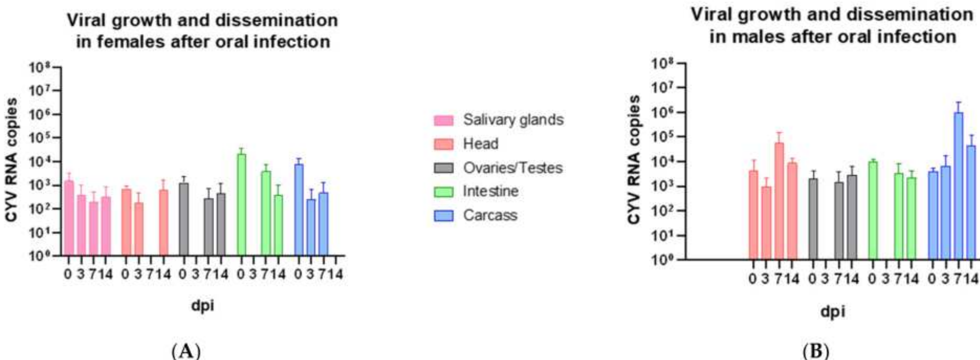

Figure 4. Growth kinetics of CYV in several organs after oral infection in *Cx. pipiens* biotype *molestus*. Mosquito dissection and preparation of different organs at zero dpi, three dpi, seven dpi, and fourteen dpi. Organs were washed three times in 1× PBS. RNA copy numbers per organ per individual are displayed per dissection day with SD. (**A**) Viral growth in females (n = 48); (**B**) Viral growth in males (n = 72). Pink—salivary glands, red—head, grey—ovaries/testes, green—intestine, and blue—body; dpi = days post-infection.

3.3. Survival

To investigate whether CYV has a negative impact on mosquito survival, we monitored the CYV-infected mosquitoes in a survival assay for 14 consecutive days and counted the surviving individuals. The survival of the female mosquitoes was not significantly impaired by CYV infection in both the injected and the oral infection group (Figure 5A,C). In male mosquitoes, a significantly reduced survival rate was observed in the CYV-injected group, with only 72% surviving 14 days after infection (Figure 5B). No significant differences were observed in the orally infected males between the control and virus-infected groups (Figure 5D).

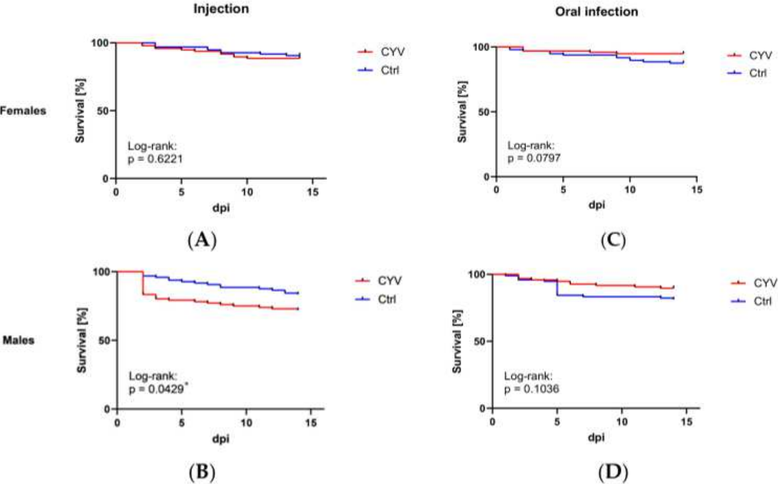

Figure 5. Survival curves of infected *Cx. pipiens* biotype *molestus* mosquitoes comparing CYV-infected and control mosquitoes per sex and infection route. The data represent the survival rates in percentages over days post-infection (dpi). (**A**) CYV-injected females vs. medium-injected females; Log-rank: $p = 0.6221$ (ns). (**B**) CYV-injected males vs. medium-injected males; Log-rank: $p = 0.0429$ (*). (**C**) CYV-fed males vs. medium-fed males; Log-rank: $p = 0.1036$ (ns). (**D**) CYV-fed females vs. medium-fed females; Log-rank: $p = 0.0797$ (ns). Red curve—CYV-infected mosquitoes; blue curve—control (Ctrl) mosquitoes.

3.4. Impact on Reproduction

To investigate whether there could be effects on reproduction in terms of egg rafts, eggs, hatching, pupation and emergence rates, these traits were analyzed based on the viruses found in the reproductive organs. No significant differences were found between CYV- and medium-injected females and their offspring for all traits tested (Table 2). When egg rafts were counted, an average of 0.66 (±0.157) egg rafts per female were laid in the virus-injected group compared with 0.59 (±0.121) egg rafts in the medium-injected females. We also found a slightly higher number of eggs per female (26.3 ± 10.48) and per egg raft (39.2 ± 10.23) in the CYV-injected females compared to the control group (21.4 ± 9.97 and 35.6 ±9.73, respectively). Upon hatching from eggs, the progeny of the medium-injected mosquitoes had higher hatching (88.2% ±9.84%), pupation (97.3 ± 3.79%), and emergence (96% ±5.39%) rates than those of the virus-injected parental generation (hatching from eggs: 76.9% ±9.13%, pupation: 88.7% ±11.48%, and emergence: 82.8% ±16.15%). The number of female offspring that emerged was also higher in the control group (55.4 ± 4.15%) than in the virus group (49.3% ± 6.34%).

Table 2. Summary of various reproductive traits comparing CYV- and medium-injected *Cx. pipiens* biotype *molestus* females with SD. For parametric data, the unpaired *t*-test was performed to determine *p*-values; *p*-values of non-parametric data were determined using the Mann–Whitney test.

Traits	CYV	Medium	*p*-Value
Egg rafts per female	0.66 (±0.157)	0.59 (±0.121)	0.4597
Eggs per female	26.3 (±10.48)	21.4 (±9.97)	0.4972
Eggs per raft	39.2 (±10.23)	35.6 (±9.73)	0.6088
Egg hatching rate (%)	76.9 (±9.13)	88.2 (±9.84)	0.0982
Pupation rate (%)	88.7 (±11.48)	97.3 (±3.79)	0.0864
Emergence (%)	82.8 (±16.15)	96.0 (±5.39)	0.1000
Percentage of females (F1) (%)	49.3 (±6.34)	55.4 (±4.15)	0.1558

3.5. Impact on Progeny

Although we could not detect CYV in the offspring, we still considered the question of whether CYV infection of the parental generation might have an impact on the development of the offspring. For this purpose, we measured the wings of the offspring of the CYV-infected mosquitoes. Examination of the morphological characteristics of the virus-affected (CYV group) and non-virus-affected (control group) progeny revealed significant differences in terms of wing sizes, which were divided into wing length (WL) and wing area (WA) (Figure 6).

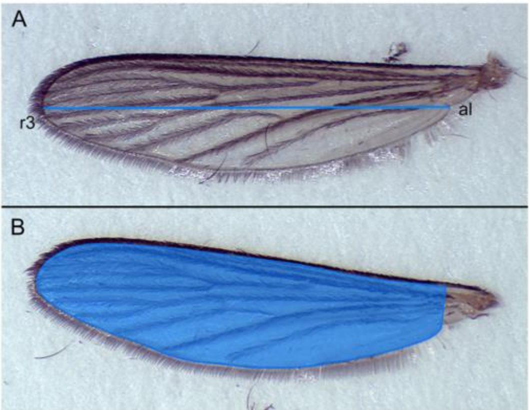

Figure 6. Measured wing traits. (**A**) Wing length was measured as the linear distance from the distal end of the alula (al) to the tip of the third radial vein (r3) at the apical margin. (**B**) Wing area was measured as the edge of the wing membrane and was limited to proximal size by a straight line forming a 90° angle with the wing rib and connecting it to the posterior edge of the alula.

With a mean length of 3.453 mm (±0.1806), the WL of the female CYV group was significantly reduced compared to the female control group (3.507 mm (±0.1934)) (Table 2). Similarly, the mean WL of the male CYV group (2.893 mm (±0.1395)) was significantly smaller than that of the control group at 2.968 (±0.1445). The measurement of WA also showed significant differences between the groups, as the WA of the female F1 generation of the control group was 2.791 mm^2 (±0.3236), whereas the females of the CYV group had a significantly smaller WA of 2.699 mm^2 (±0.2837). Males also showed a significant difference in WA between groups. The measured WA in the male control group averaged 1.851 mm^2 (±0.1715) and was, accordingly, larger than that of the CYV group with a wing area of 1.766 mm^2 (±0.1677) (Table 3).

Table 3. CYV induced alteration in the morphological trait wing size. Change in wing length (WL) and wing area (WA) with SD was defined by determining the difference for both traits between the offspring of CYV-injected females and the offspring of medium-injected females. All *p*-values are based on the Mann–Whitney test for nonparametric data. Sample size: CYV female: n = 295, CYV male: n = 286, control female: n = 102, and control male: n = 97.

	CYV		Control		*p*-Value	
	WL [mm]	**WA [mm^2]**	**WL [mm]**	**WA [mm^2]**	**WL**	**WA**
Females	3.453	2.699	3.507	2.791	0.0298	0.0162
SD	±0.1806	±0.2837	±0.1934	±0.3236		
Males	2.893	1.766	2.968	1.851	<0.0001	<0.0001
SD	±0.1395	±0.1677	±0.1445	±0.1715		

3.6. Host Specificity

3.6.1. In Vitro CYV Infection of Mammalian Cells

Furthermore, in our study, we wanted to confirm that CYV does not replicate in mammalian cells. Therefore, we inoculated BHK-21 and A459 cells with CYV and examined the cell culture supernatants at days zero, three, and seven. We could not observe CPE and, therefore, measured the RNA copies via qRT-PCR with our CYV standard. The inoculum with the infection volume of 2.5 µL in the mammalian cell infection experiments contained approximately 1.82×10^7 viral RNA copies. For the BHK-21 cells, a decrease from the day of infection with a mean of 8.31×10^3 ($\pm 4.80 \times 10^3$) RNA copies to 6.09×10^3 ($\pm 4.50 \times 10^3$) RNA copies on the seventh day was observed (Figure 7). The results regarding the A549 cells showed a mean viral RNA with 5.25×10^3 ($\pm 2.45 \times 10^3$) RNA copies at day zero to 1.02×10^4 ($\pm 9.35 \times 10^4$) RNA copies at day seven in the A549 cells. We observed high variances in these data, with RNA copies ranging from 7.41×10^2 to 2.43×10^4 in the A549 cells at day seven. Taken together, and with regard to the high variances we observed in this experiment, we do not assume that CYV would replicate in mammalian cells.

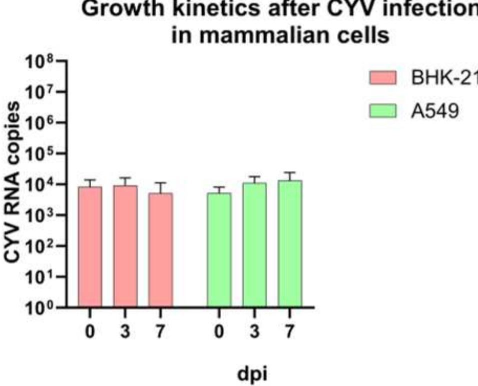

Figure 7. Viral growth kinetics of CYV in different mammalian cell lines. BHK-21 (red) and A549 (green) cells were inoculated with an infection volume of 2.5 µL of CYV displayed with SD. Samples were taken on days zero, three, and seven; dpi = days post-infection.

3.6.2. In Vivo Growth Kinetics of CYV in Other Insects

The injection of CYV into other insect species showed that the virus replicated in *Ae. albopictus*, with the highest RNA copy number on day five at 4.10×10^7 (Figure 8A), and in *D. melanogaster*, with a peak of 6.74×10^6 RNA copies on day fourteen (Figure 8B). RNA copy numbers in these species increased by up to three to four log levels over a 14-day observation period. In *A. mellifera*, the highest RNA copies were measured on days zero and three after the injection of CYV, with a mean copy number of 1.75×10^5 (Figure 8C). On subsequent sample days seven and fourteen, RNA copies decreased to 8.86×10^4 on day fourteen. In *L. migratoria*, the viral RNA copy number decreased over time, with the highest viral RNA copy number on the day of injection of 1.05×10^6 RNA copies to day 14 post-injection with the lowest copy number of 3.72×10^4 copies (Figure 8D). Altogether, we have demonstrated that CYV replicates in *Ae. albopictus* and *D. melanogaster*, both belonging to the order Diptera. In the other insect species we tested, namely, the honey bees and the locusts, CYV replication could not be measured.

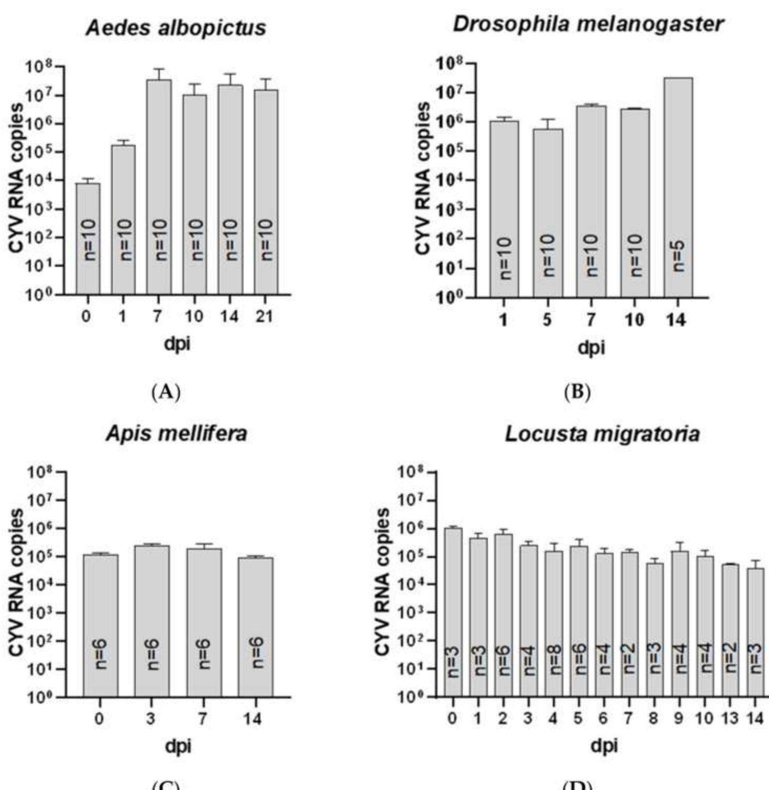

Figure 8. Viral growth kinetics in different insect species. Mean RNA copy numbers of all conducted replicates with SD are shown. (**A**) Growth kinetics in *Ae. albopictus* measured in pools with five males and five females per pool; (**B**) growth kinetics in *D. melanogaster* measured in pools with five males and five females per pool and sample day one, five, seven, and ten, and at 14 dpi only for the females; (**C**) growth kinetics in *A. mellifera* tested in pools of two bees per dpi; (**D**) growth kinetics in *L. migratoria*. Locusts were tested individually; dpi = days post-infection.

3.6.3. Survival Rates

Next, we wanted to determine whether the CYV injection had any effect on the survival of *Ae. albopictus*, *D. melanogaster*, *A. mellifera,* or *L. migratoria*. Therefore, we observed the insects for 14 days, counted the surviving individuals, and evaluated these data at the end of the experiment using Kaplan–Meier curves. The survival rates showed no significant differences between the CYV-injected and control groups with respect to *Ae. albopictus* (log-rank: $p = 0.6244$) (Figure 9A). When comparing the survival rates of the males and females with the respective control groups, again, there were no significant differences in the survival rates (for females: log-rank: $p = 0.1227$ and for males: log-rank: $p = 0.3828$). The results of the survival tests performed on the fruit flies showed a significantly lower survival rate of the CYV-injected flies compared to the control flies (log-rank: $p < 0.0001$) starting from the ninth day of fly death (Figure 9B). In the honey bees, no significant differences were observed between the control and virus-injected groups, with a *p*-value of 0.7166 (Figure 9C). No significant differences in survival rates were observed in the locusts between the CYV-injected and control groups, with a *p*-value of 0.0934 (log-rank) (Figure 9D).

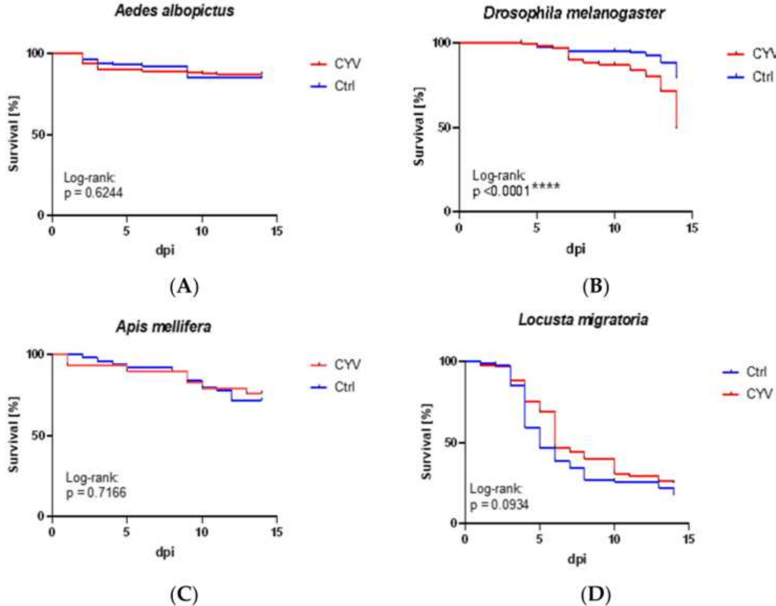

Figure 9. Survival curves. The data represent the survival rates in percentages over days post-infection (dpi). (**A**) *Ae. albobictus*: CYV-injected vs. medium-injected; Log-rank: p = 0.6244 (ns). (**B**) *D. melanogaster*: CYV-injected vs. medium-injected; Log-rank: p = <0.0001 (****). (**C**) *A. mellifera*: CYV-injected vs. medium-injected; Log-rank: p = 0.7166 (ns); two replicates. (**D**) *L. migratoria*: CYV-fed females vs. medium-fed females; Log-rank: p = 0.0797 (ns). Red curve—CYV-infected insects; blue curve—control (Ctrl) insects.

4. Discussion

Insect-specific viruses are found across a wide range of virus families, including virus families mainly known for their arbovirus representatives, such as the flaviviruses. Accordingly, most data on ISVs in mosquitoes have been obtained from these arbovirus-related ISVs, such as CFAV or CxFV. In the case of CxFV, the potential to suppress or at least delay arbovirus transmission when co-infected in cell culture with DENV and WNV was described. Furthermore, a lower rate of viral dissemination in *Ae. aegypti* mosquitoes was observed for ZIKV when co-infected with CFAV [13]. Similarly, the co-infection of WNV and the insect-specific flavivirus NHUV resulted in a reduced vector competence of mosquitoes with respect to WNV [34].

In contrast to CxFV and CFAV, less is known about CYV, which was discovered in 2010 in hibernating, free-living *Cx. pipiens* mosquitoes [17]. Following confirmation of its insect origin and phylogenetic classification in the genus Entomobirnaviruses [17], further studies investigated immunological responses to CYV infection in vitro [30,35]. Franzke et al. (2018) found that different cell cultures (Aag2, U4.4, and C7-10) are persistently infected with CYV and that infection rates vary across different passages [30]. Furthermore, the involvement of RNA interference (RNAi) in the control of CYV was confirmed through small RNA sequencing [30,35]. Similar to many other insect viruses, an inhibition of the RNAi pathway through a CYV protein was also confirmed [35]. Besides this information on immune interactions in mosquitoes, nothing is known regarding the transmission cycle of CYV nor the impact of CYV infection on mosquitoes. It is not even known if CYV is a *Culex* specific virus or if it can infect a wide range of insects.

We found that CYV was able to replicate in *Cx. pipiens* biotype *molestus* laboratory strains originating from the north of Germany after injection and oral uptake. However,

the replication rate was significantly higher after injection (Figures 1 and 4) since this infection route bypasses two important tissue barriers: the midgut infection barrier and the midgut escape barrier [36,37]. Bypassing those barriers, a systemic infection with high RNA copy numbers for CYV was established in all the tested organs. The rapid spread of CYV after injection may be due to its distribution in the mosquito body via the hemocoel. Fu et al. (1999) showed that the spread of bluetongue virus (BTV) after intrathoracic injection in *Culicoides variipennis* begins rapidly and reaches an average RNA copy number after three days [38]. Our data showed a comparable pattern without the eclipse phase or partial eclipse phase that has been described for viral dissemination after oral ingestion [39]. This phenomenon describes a decrease in viral particles within the first two days after the ingestion of a viremic blood meal in which no or at least a few viruses are detected [39]. After the oral ingestion of CYV, such an eclipse phase was observed in *Cx. pipiens* biotype *molestus*. In total, we observed reduced replication of CYV, which resulted in lower RNA copy numbers as compared to the injection. However, after an initial loss of viral RNA copies due to the digestion of the inoculum, we observed an increase in viral RNA copies in all tested organs except the salivary glands (Figure 5). The absence of replication in the salivary glands might be due to the delayed replication of CYV after oral infection. For many organs, the highest RNA copy numbers were measured at fourteen days post-infection, whereas the injection of the virus led to high viral RNA copy numbers already three and seven days after infection. In contrast to our findings, oral infection with PCV in *Cx. annulirostris* failed, whereas intrathoracic injection led to 100% infection rates [40]. For arboviruses, the dissemination of virus particles in mosquitoes after oral ingestion is well-described. Only a small number of midgut epithelial cells are thought to be susceptible to initial viral infection [36]. For ISVs, and especially for CYV, the pattern of infection in the mosquito body has not yet been described, but an initial infection of only a few cells could explain the decrease in viral RNA measured in our data on the third day after oral infection. We do not see a sex-specific dissemination pattern of CYV after feeding or injection. This result, both for viral growth in vivo after injection and after oral infection, seems logical when one considers that both males and females are constantly exposed to different ISVs in nature. When we compare this pattern, we suggest that the barriers CYV must overcome are similar to those described for arboviruses. When examining the RNA copies measured within one hour after feeding with CYV, we already found the virus in all the tested organs. This phenomenon has been observed in earlier studies in *Ae. aegypti* with Uganda S, yellow fever virus (YFV), and Semliki Forest virus and in *Ae. australis* with Whataroa virus [41,42]. Virus was detected in the hemocoel shortly after the mosquitoes fed on a viremic blood meal and, therefore, spread throughout the mosquitoes' bodies. It was hypothesized that viral particles could enter the hemolymph via an intracellular route, which could be described as a leak [43]. This so-called "leaky gut phenomenon" could be a possible explanation for the rapid distribution of CYV after feeding in our experiments. In addition, the contamination of the different organs during the process of dissection despite careful washing could account for the measured RNA copy numbers. Similar to mammals dissected without perfusion prior to dissection, contamination with a few viral particles via body fluids could account for a false-positive organ sample. False-positive organ samples can only be ruled out by the staining of the tissues with a virus specific antibody. However, such a staining procedure would only be informative at later time points of infection, as no viral protein production can be expected directly following injection.

It can be hypothesized that one possible route of CYV transmission within mosquito populations is indirect transmission via virus-contaminated food sources, as we have shown that CYV uptake by *Cx. pipiens* biotype *molestus* leads to infection. Furthermore, we were able to demonstrate that CYV is secreted in the saliva, as all the tested saliva samples were positive for CYV after injection experiments, although the measured RNA copy numbers were low. Considering that mosquitoes salivate while feeding [44], the virus could be regurgitated onto food sources and ingested by the following mosquitoes. A similar infection route via sugar meal sources has already been described for Massilia

virus, a sandfly-transmitted arbovirus, in *Phlebotomus perniciosus* [45], and experiments with *Cx. annulirostris* and *Cx. gelidus* have shown that, following infection with various flaviviruses, the virus can be delivered to sucrose food sources, albeit in varying amounts depending on the species and virus [46]. Since ISVs are unable to replicate in vertebrate cells, one possible route of transmission could be via plant food sources. However, further experiments are needed to clarify this, since we do not know if the secreted amounts of CYV are sufficient to induce infection in naïve mosquitoes

Besides contaminated plant food sources, vertical transmission if often described as a primary transmission route of ISVs. Specifically, baculoviruses and polydnaviruses rely on this mode of transmission; additionally, RNA viruses such as Sigma virus in *Drosophila* utilize this mode of transmission [47]. Regarding mosquito-associated ISVs, very little information on vertical transmission is available and solid evidence for this form of transmission only exists for three viruses: CFAV, NHUV, and Kamiti River virus (KRV). In contrast, vertical transmission is described for many arboviruses in mosquitoes (SINV, RVFV, and ZIKV). This strategy ensures viral survival even in periods between disease outbreaks, e.g., in dry seasons, when adult mosquitoes are absent [10,48–53].

For CYV, however, we were unable to demonstrate vertical transmission, although we were able to show that the virus spreads to the reproductive organs and replicates there in male and female *Cx. pipiens* biotype *molestus* mosquitoes (Figure 1). This was also described for PCV, where no vertical transmission occurred after intrathoracic injection of the virus [54]. Possible explanations for the lack of vertical transmission in our experiments could be due to the use of inbred laboratory mosquito strains or the virus exposure schemes. Another possibility could be that vertical transmission does not occur until later gonotrophic cycles; thus, the examination of the offspring of autogenous eggs laid within the first five days could be too early [55]. Anderson et al. (2008) found that the vertical transmission of WNV (after oral infection) in *Cx. pipiens* biotype *pipiens* mosquitoes does not occur until the thirteenth day after infection [56]. The results of Saiyasombat et al. (2011) also showed no vertical transmission of CxFV after the inoculation of uninfected *Cx. pipiens* biotype *molestus* mosquitoes colonized in the laboratory [50]. However, they showed that CxFV-positive females collected in the wild transmitted the virus vertically, with a prevalence of 100%. Intrathoracic injection is likely to play a minor role in preventing vertical transmission. This assumption is based on the already-successful demonstration of vertical transmission after intrathoracic injection for various bunyaviruses [57,58], ZIKV [28], YFV [59], or Japanese encephalitis virus (JEV) [60]. Vertical transmission appears to be the most obvious explanation for the prevalence of ISVs within a mosquito population, which is mainly suggested when males and females are both naturally infected [51].

Since vertical transmission does not seem to be an option for CYV, we also tested whether the virus could be transmitted horizontally by venereal transmission. The virus occurs in nature and is not an artifact of cell culture in the laboratory such as the closely related Espirito Santo virus and Drosophila X virus. Both belong to the *Birnaviridae* family but have never been found in nature in mosquitoes or fruit flies [61,62]. For CYV, we found clear evidence that the virus can survive in a mosquito population by being transmitted between the sexes. Venereal transmission has been frequently described for arboviruses (SINV, St. Louis encephalitis virus (SLEV), or ZIKV), but it has also been described for ISVs, such as Deformed Wing Virus (DWV) in honey bees, which was additionally transmitted vertically after the infection of a queen bee with DWV-positive sperm, and it has also been described for CFAV and Aedes Flavivirus (AEFV), two ISVs belonging to the *Flaviviridae* [63–68]. For the above examples, namely, SINV, SLEV, ZIKV, DWV, CFAV, and AEFV, all of these viruses can also be transmitted vertically within an insect population [48,66–70]. This contrasts with our results for CYV, wherein we found no evidence of vertical transmission, as mentioned earlier. We cannot exclude the possibility that the vertical transmission of CYV occurs naturally and that our results represent the effects of inbred mosquito strains and laboratory conditions. Apart from this, as well as the fact that we could not find another example of a virus in the literature that enables venereal but not vertical transmission, there is a need for more research in this area.

Next, the impact of a CYV infection on mosquitoes and their survival and fertility was investigated. We detected a significantly higher mortality rate for *Cx. pipiens* biotype *molestus* males in contrast to females. A selective male-killing phenomenon is already known as a possible strategy for controlling mosquito populations and has already been reported for insects via infection with bacteria, e.g., with *Wolbachia, Rickettsia*, *Spiroplasma*, *Flavobacteria*, or *Arsenophonus,* or microsporidia [71–74]. Viral male-killers were analyzed for the insect *Homona magnanima* and the oriental tea tortrix, which can be infected by Osugoroshi viruses (OGVs) [75]. CYV infection could potentially be used as a biological agent to kill males and subsequently reduce mosquito population size. In terms of vector control, this higher mortality of males could be of interest considering, on the one hand, the reduction in mosquito populations and, on the other hand, the high reproductive rate of males, who can mate and spread their genetic material for the entirety of a mosquito's life [76]. This contrasts with females, who are limited to oviposition in order to pass on their own genetic material. Furthermore, we could not find any impact on the fertility of mosquitoes if they had mated before CYV infection, although the reproductive organs are rapidly flooded and infected by the virus. The amount of F1 larvae hatched from the laid eggs as well as the pupation rate or the growth to adult mosquitoes of the offspring of the CYV-infected mosquitoes showed no significant differences compared to the control mosquitoes. Furthermore, the sex ratio—more specifically, the female ratio—of the adult F1 generation was determined. Influencing this trait, e.g., by a male bias, would affect the reproductive ability of the F1 generation. However, no significant change in the female proportion of the F1 generation was detected. There are, however, other possible traits influenced by CYV that could have an effect on the reproductive success of the offspring in terms of morphological traits, which we investigated using wing sizes. Our results show that the wing sizes of the female and male offspring of the CYV-infected mosquitoes were significantly reduced (Figure 6, Table 3). Such a decrease in wing size might suggest a decline in the fecundity of the F1 generation [77–79]; thus, the application of CYV in the parental generation might have had an impact on the reproductive success of the following generations. Since we were unable to demonstrate transovarial transmission, these changes might be due to epigenetic or immunomodulatory factors in infected ovaries. Epigenetic effects have been described in plants, mammals, and insects [80–82]. These effects include both behavioral and physiological traits that can be influenced by environmental changes or pathogenic infections and transmitted from the parental generation to the offspring [82]. In insects, phenotypic variation, transgenerational immune priming, declining insecticidal sensitivity, and the effects of diet on the offspring generation triggered by various influences on the parental generation, among others, have been described. [83–85]. We hypothesize that a CYV infection of the parental generation could affect the offspring generation, even if no transovarial transmission occurs. These effects may be phenotypic, such as the smaller wing sizes described herein, or non-phenotypic. However, further studies are needed to validate the notion that this effect negatively impacts offspring and whether non-phenotypic effects, such as immune priming or influences on offspring fertility, are also detectable by CYV infection of the parental generation. Affecting the reproductive output of mosquito colonies could promote population reduction, ultimately leading to lower population sizes.

Finally, we analyzed the host specificity of CYV. If CYV is to be used as a tool to reduce mosquito population size or to interfere with arbovirus transmission, the environmental safety of this agent needs to be confirmed to prevent unwanted impacts of the vector control measurements on other insects. We were able to confirm that the virus could not replicate in any mammalian cell culture. This finding emphasizes the assumption that CYV may be insect-specific. By examining closely and distantly related insect species, we found that CYV is not mosquito-specific but possibly diptera-specific; *Ae. albopictus*, a second tested mosquito species, seems to be unaffected by CYV, while the closely related *Drosophila* (also order Diptera) is affected by this virus (reduced survival Figure 9B). To determine whether other insect orders might be affected, we added an economically important species

from the Hymenoptera order, the honey bee, and a member of the Orthoptera order, the economically relevant agricultural pest insect *L. migratoria*. The survival rates of both the honey bee and the locust were not affected by CYV infection (Figure 9C,D), as confirmed by a lack of replication of the virus (Figure 8C,D). These results suggest that CYV may be specific to Nematocera and Brachycera but does not always exhibit pathogenic properties in these species. However, this needs to be further validated for these and other species of these two suborders.

Taken together, due to the male-killing effect and the reduced wing size, which might be associated with the reduced reproductive success of subsequent generations, CYV may be considered for use as a tool to destabilize arbovirus vector populations. Regarding the safety of the CYV agents, we were able to show that no harmful effects on economically important organisms such as honey bees could be observed.

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