Zemědělská fakulta
Faculty of Agriculture

Jihočeská univerzita v Českých Budějovicích
University of South Bohemia in České Budějovice

JIHOČESKÁ UNIVERZITA V ČESKÝCH BUDĚJOVICÍCH
ZEMĚDĚLSKÁ FAKULTA

Katedra genetiky a zemědělských biotechnologií

Disertační práce

Problematika hmyzích opylovatelů a alternativní metody kontroly houbových, bakteriálních a parazitárních onemocnění včel

Autor práce: Ing. Petr Mráz

Školitel: prof. Ing. Vladislav Čurn, Ph.D.

Školitel specialista: Ing. Martin Žabka, Ph.D.

Ing. Andrea Bohatá, Ph.D.

České Budějovice 2022

Poděkování

Tímto bych chtěl poděkovat svému školiteli prof. Ing. Vladislavu Čurnovi, Ph.D. za odborné vedení, vstřícný přístup, věcné připomínky a pomoc při zpracování této práce. Také bych chtěl poděkovat školitelům specialistům Ing. Martinovi Žabkovi, Ph.D. a Ing. Andree Bohaté, Ph.D. za cenné rady a postřehy. V neposlední řadě bych chtěl poděkovat mé rodině, která mě vždy podporovala po celou dobu mého studia.

Prohlášení

Prohlašuji, že jsem autorem této kvalifikační práce a že jsem ji vypracoval pouze s použitím pramenů a literatury uvedených v seznamu použitých zdrojů.

Prohlašuji, že jsem doktorskou disertační práci vypracoval samostatně na základě vlastních zjištění a za pomoci uvedené literatury.

Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své disertační práce, a to v nezkrácené podobě elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. Zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

V Českých Budějovicích dne ....

Ing. Petr Mráz

Abstrakt

Tato disertační práce se zabývá významem hmyzích opylovatelů a upozorňuje na jejich úbytek vlivem intenzivního zemědělství. Velká pozornost je věnována včele medonosné jakožto hlavnímu opylovateli, především otázce kvalitní výživy včelstev v návaznosti na podpoření detoxikace pesticidů. Dále je pozornost věnována alternativním možnostem kontroly vybraných včelích patogenů a průzkumem jejich prevalence v České republice. Práce je rozdělena na dvě hlavní části: literární rešerši a experimentální část složenou ze šesti podkapitol s výsledky z vlastní výzkumné činnosti.

První studie pojednává o vlivu opylení na kvalitativní a kvantitativní parametry výnosu zimolezu modrého. Bylo testováno několik variant opylení a nejlepšího výsledku ve všech sledovaných parametrech bylo dosaženo opylením přirozenými opylovateli. Varianty ruční opylení a bez opylení způsobovaly nerovnoměrné dozrávání i nižší výnos. Dále byla hodnocena diverzita a početnost opylovatelů v blízkosti této plodiny a zjišťovány nejvhodnější opylovatelé, kterými se zdají být čmeláci a včela medonosná.

Druhá studie řeší vliv intenzity zemědělství na diverzitu a početnost opylovatelů v krajině. Srovnávány byly lokality s ekologickým a konvenčním režimem hospodaření. Významně vyšší diverzita i početnost opylovatelů byla zaznamenána na lokalitě obhospodařované ekologickým zemědělstvím. Kromě toho byla také sledována kontaminační zátěž rezidui pesticidů ve včelách. Na lokalitě s konvenčním zemědělstvím byla detekována rezidua několika pesticidů, zatímco na lokalitě s ekologickým zemědělstvím nebyla detekována žádná.

Třetí studie se zabývá vlivem výživy, konkrétně fenolických látek, na schopnost včel detoxikovat pesticid, kterému byly vystaveny. V provedeném experimentu byly včely v klíckách krmeny směsí vybraných polyfenolů, běžně se vyskytujících v pylu, a pesticidem thiaclopridem. Po dobu 14 dnů byla sledována mortalita, denní spotřeba krmiva a ve stanovených intervalech probíhala analýza míry exprese detoxikačních genů. Byl prokázán pozitivní vliv fenolických látek na délku života intoxikovaných včel, stejně jako vyšší spotřeba krmiva, což může indikovat zvýšenou potřebu těchto látek. Naopak zvýšená míra exprese detoxikačních genů potvrzena nebyla.

Další studie se věnují patogenům včely medonosné. Jedna z nich sleduje výskyt a prevalenci vybraných hlavních včelích patogenů na území České republiky a porovnává odlišné typy habitatu, jako jsou městské oblasti, zemědělsky intenzivně obdělávané oblasti a chráněná přírodní oblast. Překvapivě nejčastěji byl detekován patogen *Lotmaria passim*. Z virových onemocnění pak DWV komplex a ABPV. Obecně se více eukaryotních patogenů vyskytovalo ve městech a zemědělské krajině. Naopak více virových onemocnění bylo zaznamenáno v chráněné přírodní oblasti.

Pátá podkapitola se skládá ze čtyř publikací a zabývá se využitím rostlinných silic ke kontrole roztoče *Varroa destructor* a entomopatogenní houby *Ascosphaera apis*. První publikace porovnává růst a vývoj houby *A. apis* na různých kultivačních mediích a navrhuje nové medium s přídavkem včelího plodu, na kterém byla zaznamenána největší sporulace. Další dvě publikace pojednávají o fungicidním účinku vybraných rostlinných silic v laboratorních podmínkách. Nejlepší výsledky vykazovaly silice z tymiánu, cedrového dřeva, hřebíčku a skořice. Čtvrtá publikace se věnuje akaricidnímu účinku vybraných rostlinných silic na roztoče *V. destructor* a zároveň hodnotí toxicitu těchto silic na dospělé včely. Na základě těchto výsledků byly stanoveny rostlinné silice s největším poměrem LD_{50} na včely / LD_{50} na roztoče (selectivity ratio), které mají největší potenciál využití ve včelařské praxi. Jedná se o silice z manuky, máty peprné, dobromysli a vavřínu kubébového (litsea).

Poslední kapitola řeší kontrolu původce onemocnění moru včelího plodu, bakterii *Paenibacillus larvae*, pomocí enzymů trávicího traktu zavíječe voskového. Larvy zavíječe byly krmeny voskovými mezistěnami kontaminovanými bakterií *P. larvae*. Následně byl jejich zažívací trakt rozdělen na tři části, ze kterých proběhla kultivační i molekulární detekce bakterie. V předních částech traktu byla bakterie detekována, ale v zadní již ne. To může naznačovat sporicidní účinek trávicích enzymů, nebo pomalý průchod spor zažívacím traktem. Pro upřesnění jsou však zapotřebí další experimenty.

Klíčová slova: Včela medonosná; opylovatelé; screening včelích patogenů; *Ascosphaera apis; Paenibacillus larvae; Varroa destructor;* polyfenoly; rostlinné silice

Abstract

This Ph.D. thesis is focused on the importance of insect pollinators and pointed out to their loss due to intensive agriculture. Great attention is paid to the honey bee as the main pollinator, especially to the issue of quality nutrition of bee colonies in connection with the support of detoxification of pesticides. Furthermore, the main effort is devoted to alternative possibilities of control of selected bee pathogens and research of their prevalence in the Czech Republic. The work is divided into two main parts: a detailed background research and an experimental part consisting of six subchapters with results from my own research studies.

The first study deals with the effect of pollination on the qualitative and quantitative yield parameters of honeysuckle. Several pollination variants were tested and the best result in all monitored parameters was achieved by the pollination with natural pollinators. Variants of manual pollination and without pollination caused uneven maturation of fruits and lower yields. Furthermore, the diversity and abundance of pollinators in the vicinity of this crop were observed and the most suitable pollinators identified which appear to be bumblebees and the honey bee.

The second study deals with the impact of agricultural intensity on the diversity and abundance of pollinators in the landscape. Localities with organic and conventional management regimes were compared. Significantly higher diversity and abundance of pollinators was recorded in the locality managed by organic farming. In addition, the contamination load of pesticide residues in bee's body was also monitored. Residues of several pesticides were detected at the site with conventional agriculture, while none of them were detected at the site with organic farming.

The third study examines the effect of nutrition, specifically phenolic substances, on the ability of bees to detoxify the pesticide to which they have been exposed. In the experiment, the bees in the cages were fed with a mixture of selected polyphenols, commonly found in pollen, and the pesticide thiacloprid. Mortality and daily feed consumption were monitored for 14 days, and the expression level of detoxification genes was analyzed at specified intervals. Phenolic substances have been shown to have a positive effect on the lifespan of intoxicated bees, as well as higher feed consumption, which may indicate an increased need for these substances. In contrast, the increased expression of detoxification genes was not confirmed.

Other studies focus on honey bee pathogens. One of them monitors the occurrence and prevalence of selected major bee pathogens in the Czech Republic and compares different types of habitats, such as urban areas, agriculturally intensively cultivated areas and protected natural areas. Surprisingly, the most often detected pathogen was *Lotmaria passim*. From the viral diseases, the highest rate of occurrence had DWV complex and ABPV. In general, more eukaryotic pathogens were found in cities and agricultural landscapes. On the contrary, more viral diseases were recorded in the protected natural area.

The fifth subchapter consists of 4 publications and deals with the use of essential oils to control the *Varroa destructor* mite and the entomopathogenic fungus *Ascosphaera apis*. The first publication compares the growth and development of the fungus *A. apis* on different culture media and proposes a new medium with the addition of bee brood, on which the greatest sporulation was recorded. Another 2 publications deal with the fungicidal effect of selected essential oils in laboratory conditions. The best results were shown by essential oils of thyme, cedar wood, cloves and cinnamon. The fourth publication deals with the acaricidal effect of selected essential oils on the *V. destructor* mites and at the same time evaluate the toxicity of these oils to adult bees. Based on these results, essential oils with the highest LD_{50} to bees / LD_{50} to mites ratio (selectivity ratio) were determined, which have the greatest potential for use in beekeeping practice. These are essential oils of manuka, peppermint, oregano and litsea.

The last chapter deals with the control of the causative agents of American foulbrood, the bacterium *Paenibacillus larvae*, by the enzymes of the digestive tract of the wax moth. The larvae of the moth were fed with wax foundation contaminated with *P. larvae*. Subsequently, their digestive tract was divided into 3 parts, from which culture and molecular detection of bacteria were carried out. Bacteria were detected in the anterior tract, but not in the posterior tract. This may indicate a sporicidal effect of digestive enzymes, or a slow passage of spores through the digestive tract. However, further experiments are needed for clarification.

Keywords: Honey bee; Pollinators; Screening of Honey Bee Pathogens; *Ascosphaera apis*; *Paenibacillus larvae*; *Varroa destructor;* Polyphenols; Essential oils

Obsah

1 Úvod

Kromě poskytování mnoha včelích produktů leží hlavní význam včely medonosné (*Apis mellifera*) v opylovací činnosti, jelikož patří mezi nejvýznamnější opylovatele zemědělských plodin i planě rostoucích rostlin. Její kosmopolitní rozšíření a vysoká početnost zajišťují relativně stabilní výnosy a významně se podílí na potravinové bezpečnosti. Některé druhy plodin jsou přímo závislé na kvalitním opylení a včelstva jsou k nim pravidelně přesouvána, mnohdy i na velké vzdálenosti. Jde především o mandloňové a jabloňové sady v USA, ale s kočováním včelstev k zemědělským plodinám se lze často setkat i v podmínkách České republiky. V poslední době se však potýkáme s plošnými úhyny včelstev stále častěji. Příčiny úhynů nejsou zcela známy, ale pravděpodobně se na nich podílí hned několik faktorů najednou. Nejvýznamnějšími faktory se zdají být včelí onemocnění, kontaminace životního prostředí a včelích produktů pesticidy, případně dalšími xenobiotiky, dále nedostatečná výživa, změny krajinného rázu, klimatické změny, nebo i kočování se včelstvy. Na mnoha místech tak dochází k úbytku jak včely medonosné, tak i dalších hmyzích opylovatelů. To může negativně ovlivňovat nejen potravinovou bezpečnost vlivem nedostatečného opylení, ale i ekosystémové služby, diverzitu rostlin, trofické vazby a vést až k narušení stability ekosystémů.

Z výše uvedených důvodů je problematika týkající se zdravotního stavu včely medonosné velmi aktuální a dostává se do popředí pozornosti jak fundovaných odborníků, tak i laické veřejnosti. V této souvislosti byla disertační práce zaměřena na zlepšení zdravotního stavu včelstev pomocí nových, alternativních terapeutických postupů zdolávání vybraných onemocnění včel. Dále se zabývá vlivem výživy na schopnost detoxikace pesticidů a délku života včel. V dnešní době, kdy aplikace pesticidů neustále stoupá, je téměř nemožné vyhnout se jejich reziduím. Proto je nyní velmi důležité porozumět detoxikačním mechanismům včel. Ve většině případů jsou pesticidní přípravky před uvedením na trh testovány pouze jednotlivě, což nemusí na včely působit akutně, ale oslabuje je chronicky. V případě nedostatečné výživy, často způsobené pěstováním monokultur na rozsáhlých plochách, je však detoxikace včel omezována a i chronické působení může vést ke značným oslabováním kolonií, případně až k jejich kolapsu. Do polí jsou však zřídka aplikovány pesticidy

jednotlivě. Většinou se jedná o směs několika pesticidů zaměřených na různé patogeny. Tyto směsi mohou přestavovat velké nebezpečí, protože často působí synergicky a zvyšují tak svůj negativní dopad na opylovatele. Dokonce většina přípravků k potlačování nejvýznamnějšího včelího onemocnění, varroózy, je pesticidního charakteru a donedávna byla jejich aplikace zákonem nařízena. Nyní se situace zlepšuje a zejména u mladších generací včelařů je snaha používat k přírodě šetrnější látky, jako jsou např. organické kyseliny nebo rostlinné silice.

Hlavním tématem práce bylo stanovit nové terapeutické postupy zdolávání vybraných včelích onemocnění na základě využití rostlinných silic, nebo trávících enzymů zavíječe voskového (*Galleria mellonella*) a dále vyhodnotit vliv stravy, konkrétně polyfenolů, na detoxikační schopnosti včely medonosné. Také byla sledována diverzita a početnost včel a včelích patogenů v odlišných habitatech s různou měrou pesticidní a antropogenní zátěže a v neposlední řadě význam opylení, zejména kvantitativní a kvalitativní parametry výnosu zimolezu modrého (*Lonicera caerulea*) v závislosti na různém typu opylení.

2 Literární přehled

2.1 Význam včely medonosné

Včela medonosná (*Apis mellifera* L.) je považována za jedno z nejdůležitějších hospodářských zvířat. Lidstvo využívá její produkty již od starověku, zejména včelí med, což dokládá jeho vysokou nutriční hodnotu a zdravotní nezávadnost. Mezi další využívané včelí produkty z této doby patří včelí vosk, který byl nepostradatelnou surovinou pro výrobu svící. Později se začal využívat také propolis jako antimikrobiální složka. V současné době jsou populární také fermentovaný pyl, tzv. perga, mateří kašička a včelí jed, využívané zejména v potravinářském, kosmetickém a farmaceutickém průmyslu. To dělá z včelích produktů velmi důležité a cenné suroviny (Cianciosi et al., 2018; Ruoff and Bogdanov, 2004). Jejich léčivých účinků je využíváno po staletí (Nolan et al., 2019) a v poslední době popularita včelích produktů v medicíně ještě vzrůstá, zejména kvůli zvyšující se rezistenci patogenů k antibiotikům a oblibě přírodních produktů (Cornara et al., 2017; Jagua-Gualdrón et al., 2020; Lu et al., 2019).

Největší význam má však včela medonosná pro svou opylovací činnost. Jelikož má celosvětové rozšíření a vysokou početnost, řadí se mezi hlavní opylovatele jak zemědělských plodin, tak i planě rostoucích rostlin. Opylování umožňuje pohlavní rozmnožování rostlin a zajišťuje tak vysokou genetickou i druhovou diverzitu flory. Opylování je základem i zemědělské produkce, zajišťující potravinovou bezpečnost. Kvalitní opylení přispívá k větším výnosům i vyšší kvalitě produktů (Aizen et al., 2009; Gallai et al., 2009). Některé zemědělské podniky jsou dokonce přímo závislé na přísunu včelstev k pěstovaným plodinám za účelem opylení, zejména mandloňové a jabloňové sady (Smart et al., 2019).

2.2 Ohrožení včely medonosné a související dopady

V současné době jsou však včely vystaveny mnoha stresovým faktorům, zejména podvýživě způsobené redukcí biodiverzity kvetoucích rostlin, v extrémních případech vedoucích až k monodietě (Dolezal et al., 2019; Smart et al., 2019). Další stres včelám způsobují časté přesuny na dlouhé vzdálenosti, nevhodné nebo nedostatečné zásahy včelařů (Biesmeijer et al., 2006) a především, aplikace fungicidů, akaricidů a antibiotik do úlového prostoru a aplikace mnoha pesticidů v zemědělství, jež mohou působit synergicky a zvyšovat tak svůj negativní účinek

(Iwasa et al., 2004; Thompson et al., 2014). Kromě toho, pesticidy (Motta et al., 2018) a antibiotika (Raymann et al., 2017) negativně ovlivňují střevní mikrobiom včel, který se podílí na získávání živin z potravy, pozitivně ovlivňuje produkci včelích hormonů a bílkovin a v neposlední řadě významně podporuje imunitu včel (Kwong et al., 2017; Zheng et al., 2017). Všechny tyto faktory dělají včely více náchylné k parazitům a patogenům způsobujícím různá onemocnění, která by včelstva za nestresujících podmínek byla schopna překonat. Proto nyní včelstva trpí parazity a patogeny častěji než dříve a vzrůstají obavy, jak zabránit narůstajícím případům kolapsů včelstev (Genersch, 2010a; Goulson et al., 2015; Kulhanek et al., 2017), stejně jako dalších hmyzích opylovatelů (Hallmann et al., 2017; Potts et al., 2010).

Jelikož včely dokáží pomocí jemných chloupků na těle zachytit i velmi malé množství kontaminantů v krajině, ať už jde o rezidua pesticidů, těžké kovy, radionuklidy nebo polycyklické aromatické uhlovodíky, jsou často využívány jako bioindikátor životního prostředí. Častější plošné kolapsy včelstev tak mohou naznačovat i zvyšující se kontaminační zátěž životního prostředí (Porrini et al., 2003).

Velký problém současného včelařství tedy představují stále četnější plošné kolapsy včelstev vedoucí k nedostatečné produkci včelích produktů a jejich nabídka je převyšována poptávkou (Biesmeijer et al., 2006). To vede k rozsáhlému falšování včelích produktů (Sahlan et al., 2019). Nejvíce je takto ovlivněn med, který je navíc ve velkém importován do Evropy a USA ze vzdálených oblastí, např. Číny. Tyto medy obsahují odlišná pylová zrna, která mohou způsobovat alergické reakce. V některých případech obsahují také rezidua pesticidů a antibiotik, které jsou v Evropské Unii zakázány (Bargańska et al., 2011; Ruoff and Bogdanov, 2004). Včelí vosk je také často kontaminován pesticidy, a to jak v Evropě (Morales et al., 2020; Vázquez et al., 2015) a USA (Frazier et al., 2008), tak i v Africe (Llorens-Picher et al., 2017). Tato kontaminace má za následek zvýšení poptávky po kvalitním vosku, kterého je nedostatek. Proto je včelí vosk často nastavován parafinem a stearinem, což způsobuje další problémy v chovu včel (Svečnjak et al., 2019).

Ještě větší obavy však vzbuzuje úbytek opylovatelů v krajině, který se souhrnně označuje jako opylovací krize. V přírodních ekosystémech dochází vlivem nedostatku opylovatelů k narušení ekosystémových služeb, trofických řetězců i redukci biodiverzity (Aizen et al., 2009; Gallai et al., 2009; Hallmann et al., 2017).

Naopak plochy pěstovaných zemědělských plodin závislých na hmyzím opylení se každoročně zvyšují a nedostatečné opylení způsobuje nestabilní výnosy a snižuje kvalitu produkovaných komodit, což vede ke značným ekonomickým ztrátám (Aizen et al., 2009; Lippert et al., 2021) a ohrožuje potravinovou bezpečnost (Klein et al., 2007). Hmyzosnubné zemědělské plodiny jsou navíc hlavním zdrojem mikronutrientů pro lidskou výživu, zatímco větrosnubné plodiny jsou spíše zdrojem kalorií. Nedostatečné opylení tedy může přispívat k deficitu těchto důležitých živin a vést k podvýživě (Eilers et al., 2011). Výnosy některých zemědělských plodin jsou limitovány pouze kvalitním opylením (Reilly et al., 2020). V určitých oblastech se proto využívá umělého opylení sadů, kde pracovníci štětečky opylují jednotlivé květy. Cena lidské práce je však obecně velmi vysoká a neustále roste, což v tomto případě způsobuje i rostoucí náklady na produkci potravin (Partap and Ya, 2012). Alternativu představují robotické drony, které s sebou ale nesou velké množství rizik, jako např. nízká efektivita opylení, vysoká pořizovací cena, riziko destabilizace ekosystémů a ekosystémových služeb a v neposlední řadě větší potravinová nejistota (Potts et al., 2018). Ani umělé opylení rostlin však neposkytuje takové výnosy a kvalitu plodů jako v případě opylení přirozenými opylovateli (Chautá-Mellizo et al., 2012).

2.3 Přirozené obranné mechanismy včel

Včely mají několik obranných mechanismů. První z nich je kutikula, vnější nebuněčná vrstva složená převážně z chitinu, sacharidů a vosků, která slouží jako mechanická i biochemická bariéra zabraňující mikrobiální invazi. Po narušení této bariéry dochází k aktivaci vrozené imunity založené na nespecifické buněčné a humorální aktivitě (Tsakas and Marmaras, 2010). Buněčná imunita je spojená s hemocyty a zahrnuje fagocytózu, nodulaci a enkapsulaci různých patogenů, jako jsou bakterie, kvasinky, viry, protozoa a nematoda. Hemocyty jsou buňky přenášené v hemolymfě se specifickými povrchovými útvary, kterými se přichytávají na různé povrchy. To je důležité při nodulaci a enkapsulaci patogenů a cizorodých látek (Marringa et al., 2014; Negri et al., 2013). Mále cizorodé struktury mohou být pohlceny fagocytózou, zatímco u větších dochází ke kooperaci více hemocytů, které tyto částice obklopí a eliminují (Dubovskiy et al., 2016). Množství hemocytů se

snižuje s věkem včely (Schmid et al., 2008), ale jejich účinek zůstává stále stejný (Wilson-Rich et al., 2008).

Mezi další imunitní reakce patří melanizace, představující kombinaci buněčných a humorálních procesů, které vznikají během enkapsulace a nodulace cizorodých struktur. Při melanizaci dochází k eliminaci negativních vlivů patogenů, nebo jejich zmírnění, například zabráněním ztráty hemolymfy a vniknutí dalších mikroorganismů (Larsen et al., 2019). Celý proces se nazývá fenoloxidázová kaskáda a tvoří jednu ze základních součástí vrozené imunity včel. Tomuto procesu předchází rozpoznání cizorodých struktur (PAMPs – pathogen-associated molecular patterns) a následná indukce adheze hemocytů na cílové struktury. Tím se z hemocytů aktivuje produkce monomeru profenoloxidázy, jehož štěpením vzniká katalyticky aktivní enzym fenoloxidáza. Při vlastní enkapsulaci vznikají jako meziprodukty vysoce reaktivní látky na bázi kyslíku (H_2O_2, $\bullet O_2$) a dusíku (NO•), které mají značnou cytotoxickou aktivitu vůči mikroorganismům (Negri et al., 2012).

Druhým typem vrozené imunity je humorální imunita, která představuje nejdůležitější obranný mechanismus včely jako jedince. Je založená na cílené sekreci antimikrobiálních peptidů (defensin, abaecin, apidaecin a hymenoptaecin) a dalších biologicky aktivních látek (např. lektinů, lysozomů a fenoloxidázy) po prvotní detekci mikrobiální infekce. Jedná se o malé proteiny složené z 12-50 aminokyselin, které jsou z velké části produkovány v tukovém tělese a hemolymfou transportovány na místo začínající infekce, kde eliminují nebo neutralizují potenciální patogeny (Larsen et al., 2019). Tato imunitní odpověď však značně zatěžuje organismus, což vede ke snížení délky života (Moret and Schmid-Hempel, 2000) a omezení kognitivních funkcí (Alghamdi et al., 2008; Mallon et al., 2003). Oba typy vrozené imunity, jak celulární, tak i humorální, jsou v případě včely medonosné méně rozvinuté než u ostatního hmyzu. Důkazem je celkově méně genů spojených s imunitou, z toho pouze jediný gen kódující profenoloxidázu (Evans et al., 2006; Lourenço et al., 2013), nebo 4 skupiny antimikrobiálních peptidů (Larsen et al., 2019) v porovnání s 10-15 skupinami u většiny hmyzích druhů (Cooper, 2006).

Včely mají však výhodu sociálního života, což jim umožňuje využívat sociální imunitu. Jejím principem je vyhýbání se patogenům a omezování jejich šíření. Jako prevenci využívají včely rostlinné pryskyřice, které zpracovávají na propolis a přidávají do vosku nebo s ním tmelí vnitřní povrch úlu (Simone et al., 2009). Propolis vytváří antimikrobiální prostředí, které brání růstu bakteriálním

(Borba and Spivak, 2017) i houbovým patogenům (Alotaibi et al., 2019; Arismendi et al., 2018; Wilson et al., 2017). Další sofistikovaný systém ve včelstvu je prostorové rozdělení různých kast. Mladé včely pečující o plod mají omezený kontakt s létavkami, které jsou vystavovány vyššímu riziku infekce (Stroeymeyt et al., 2014). Jakmile dojde k infekci uvnitř včelího úlu, dospělé včely rozpoznají nakažený plod a za účelem omezení šíření patogenů jej vyhazují. Tato aktivita se označuje jako hygienické chování a patří k nejdůležitějším faktorům sociální imunity (McAfee et al., 2018). Kromě plodu je možné vyhazovat i dospělé nemocné včely (Baracchi et al., 2012), které ale vzhledem k jejich altruistickému chování ve většině případů opouštějí včelstvo samy (Rueppell et al., 2010). Mezi další formy sociální imunity patří tzv. "sociální horečka" (social fever). Vyznačuje se cíleným zvýšením teploty na takovou hodnotu, které je pro parazita/patogena letální, ale včely ji ještě snesou. Tato změna teploty bývá patrná zejména v oblasti včelího plodu (Goblirsch et al., 2020; Starks et al., 2000). Zanedbatelný není ani vliv matky, která se páří s několika trubci (polyandrie), což jí umožňuje zvýšit genetickou variabilitu své kolonie. V úlu se tedy vyskytují včely s různými otci. V praxi to znamená, že každá tato linie má jinou náchylnost k parazitům a patogenům, což zpomaluje šíření onemocnění (Desai and Currie, 2015; Seeley and Tarpy, 2007; Tarpy, 2003).

2.4 Studovaná včelí onemocnění

2.4.1 Varroóza

Za nejnebezpečnějšího včelího patogena je považován parazitický roztoč kleštík včelí (*Varroa destructor* Anderson and Trueman). Nadměrnou infestací ve včelstvu tento roztoč způsobuje onemocnění zvané varroóza (Rosenkranz et al., 2010). Do roku 2000 byl označován jako *Varroa jacobsoni,* poté však došlo k jeho reklasifikaci na základě rozdílných haplotypů (Anderson and Trueman, 2000). Tento roztoč je původem z východní Asie, kde parazituje na včele východní (*Apis ceranae*). Evolučním vývojem si včela východní vytvořila řadu obranných mechanizmů a proto je tento vztah parazita a hostitele v rovnováze (Lin et al., 2018; Wang et al., 2020). Ve druhé polovině minulého století však došlo k přenosu kleštíka včelího na včelu medonosnou a jeho rozšíření téměř po celém světě. Pro včelu medonosnou je to zcela nový patogen, proti kterému nemá vytvořené dostatečně účinné obranné mechanizmy a snadno mu podléhá. Pro ochranu svých včelstev

začali chovatelé používat různá ošetření, zejména syntetickými akaricidy, která sice redukují počty roztočů ve včelstvech, ale také znemožňují včelstvům adaptovat se a vytvořit si specifické obranné mechanizmy (Guichard et al., 2020; Locke, 2016; Panziera et al., 2017).

Zásadním rozdílem mezi původním hostitelem včelou východní a novým hostitelem včelou medonosnou je omezení reprodukce roztoče pouze na trubčím plodu. Mezi další přirozené obranné mechanismy redukující počty roztočů patří např. hygienické chování včel, kterému se přikládá největší význam a je nejvíce studovaným mechanismem redukujícím počty roztočů (Traynor et al., 2020). Do této kategorie patří varroa senzitivní hygiena vyznačující se odstraňováním plodu napadeného roztoči (Panziera et al., 2017). Včelstva prošlechtěná na vysokou úroveň této vlastnosti preferují čištění takových buněk, které jsou napadeny více roztoči najednou, čímž významně omezují reprodukci a další šíření roztočů (Kim et al., 2018). Hygienické chování ale pravděpodobně není závislé na přítomnosti roztoče v plodové buňce, jako spíše na poškození včelího plodu, tzn. včelí plod parazitovaný více roztoči, nebo roztoči s více virulentní variantou virů je odstraňován přednostně z důvodu většího poškození včelí larvy (Schöning et al., 2012). Z poškozených nebo mrtvých larev přes perforované víčko buňky procházejí specifické látky, jako např. kyselina olejová, které stimulují včely k odstraňování poškozeného plodu (McAfee et al., 2018), nebo jeho kontrolu odvíčkováním buněk (Martin et al., 2019). Při otevření buněk s parazitovaným plodem dojde k přerušení reprodukčního cyklu roztoče, ale samička často unikne ven a může se dále reprodukovat (Rosenkranz et al., 2010). Tomu lze zabránit vhodným středně až dlouhodobým ošetřením. Roztoči v zavíčkovaných buňkách jsou totiž ve většině případů chráněni před účinnou látkou. Jejich vyrušením při odvíčkování buňky a následném útěku však dojde k požadovanému kontaktu s účinnou látkou. Dalším mechanismem redukující počty roztočů je zvýšená náchylnost včelích larev na toxiny, které roztoč vylučuje slinami do svého hostitele. V tomto případě může dojít k usmrcení hostitele dříve, než proběhne vývojový cyklus roztoče, což limituje jeho reprodukci. K tomuto jevu dochází na původním hostiteli – včele východní. Mrtvé larvy dále stimulují hygienické chování včel (Page et al., 2016; Zhang and Han, 2018). Velmi účinný může být také tzv. “grooming“, neboli cílené odstraňování parazitujících roztočů na tělech včel pomocí kusadel a předního páru nohou (Pritchard, 2016).

Patogeneze

Parazitující roztoči se živí lipidovou složkou včelího plodu i dospělců (Ramsey et al., 2019), čímž včely oslabují, významně snižují jejich imunitu i celkovou hmotnost (Duay et al., 2003) a omezují jejich letové schopnosti (Duay et al., 2002; Kralj and Fuchs, 2006). Trubci, kteří byli parazitováni během svého vývoje, produkují v dospělosti výrazně méně spermií (Duay et al., 2002). Byla také prokázána významně kratší délka života včel (Amdam et al., 2004). Včely po vylíhnutí prochází různými obdobími, ve kterých vykonávají odlišné činnosti v úlu. Od 12-15 dne se stávají létavkami a vydávají se ven z úlu shánět potravu. Pokud však byly včely během svého vývoje parazitovány roztočem, tato jednotlivá období se zkracují a z úlu vylétávají za potravou mnohem dříve. Jejich mozkové funkce za tak krátkou dobu ještě nejsou zcela vyvinuté a to včelám způsobuje značné problémy s učením, pamětí i orientací (Gómez-Moracho et al., 2017), a omezuje adaptaci létavek na nové podmínky prostředí (Muijres et al., 2020). Ve výsledku tyto včely tráví výrazně více času mimo úl a mají problémy s úspěšným návratem do své mateřské kolonie, což indikuje i navigační potíže (Kralj and Fuchs, 2006). Kromě toho se omezuje přísun potravy do včelstva. Tento stav iniciuje vylétávání ještě mladších včel, avšak se stejným efektem. Ve výsledku to může vést až ke kolapsu celého včelstva (Gómez-Moracho et al., 2017). Tyto neurologické změny byly také potvrzeny změnou expresí některých genů ovládajících správnou funkci mozku (Morfin et al., 2020). Často na včelstva působí i další negativní faktory ve stejný okamžik spolu s roztočem *Varroa* a u některých těchto kombinací byl dokonce pozorován synergický efekt, tzn. výsledný efekt je větší než součet obou negativních faktorů. S tímto jevem se lze setkat u pesticidů (Annoscia et al., 2020; Schwartz et al., 2020), dalších patogenů (Cox-Foster et al., 2007; Higes et al., 2008), nebo v souvislosti se změnami klimatu (Le Conte and Navajas, 2008).

Nejvíce však roztoč poškozuje včelstva jako vektor různých včelích virů, čímž usnadňuje jejich přenos a výrazně zvyšuje výskyt ve včelstvech. Přenos virů se uskutečňuje na základě tří kritérií. První z nich je krmení se na virem infikovaném jedinci. Dalším krokem je transport roztoče na jinou včelu a na konec přenos, případně získání nových virových částic. Nejčastěji jsou přenášeny virus deformovaných křídel (*Deformed wing virus* – DWV) a virus akutní paralýzy včel (*Acute bee paralysis virus* – ABPV), které jsou zároveň nejčastějšími a nejnebezpečnějšími viry vyskytující se ve včelstvech (Posada-Florez et al., 2020).

Úspěšnost a efektivita přenosu závisí na mnoha faktorech, jako je přítomnost viru v hostiteli, schopnost přežívat v necílovém organismu (v roztoči), případně se v něm i replikovat, nebo odolnost včely vůči danému viru. Právě schopnost viru replikovat se i v roztoči *Varroa* je velmi zásadní a byla objevena u viru DWV-B (Gisder and Genersch, 2021), dříve označovaném jako VDV-1 (*Varroa destructor* virus-1), protože byl izolován právě z roztočů *Varroa* (Ongus et al., 2004). Bylo prokázáno, že pokud roztoč obsahuje více virových částic, je schopen vyvolat u vyvíjejících se včel akutní průběh virových onemocnění vedoucích k zimním úhynům včelstev (Gisder et al., 2009). Přechod viru na nového hostitele, je často doprovázen změnou virulence, která je v tomto případě vyšší než u klasické varianty DWV-A (Gisder et al., 2018; McMahon et al., 2016; Natsopoulou et al., 2017). Kromě toho se jeví vztah roztoče a viru jako symbiotický, protože DWV potlačuje imunitu včel a podílí se na posílení reprodukce roztoče (Di Prisco et al., 2016).

V případě ponechání infestovaných včelstev v mírném klimatickém pásmu bez zásahů včelaře dojde po exponenciálním rozmnožení roztočů během letního období k maximu roztočů ve včelstvu na podzim, kdy již matky za běžných podmínek zastavují kladení. Při velkém oslabení včelstva vlivem varroózy však matka stále klade vajíčka s cílem zachránit kolonii. Výsledek je ale opačný, protože nový včelí plod umožňuje pokračování reprodukce roztoče a tento stav bývá pro včelstva fatální (Kang et al., 2016; Messan et al., 2021). Ke značným problémům však může dojít i po řádném ošetření včelstev, a to tzv. reinvazí roztočů, kdy silně infestovaná včelstva začnou kolabovat a včely ze silnějších kolonií loupící cukerné zásoby s sebou do mateřských kolonií přenášejí i velké množství roztočů (Peck and Seeley, 2019), zejména pokud jde o včelstva na stejném stanovišti nebo v jeho těsné blízkosti (Nolan and Delaplane, 2017). Kromě maximálního počtu roztočů ve včelstvu dochází zároveň v tomto období ke snižování populace včel, tzn., že na jednu včelu připadá v poměru více roztočů. Právě proto se ve včelstvech objevuje i více klinických příznaků onemocnění (Traynor et al., 2020). Mezi typické klinické příznaky varroózy patří velká mezerovitost plodu, zmrzačené včely, zejména s deformovanými křídly, častá výměna matek nebo bezmatečnost a rapidní úbytek včel. Z těchto důvodů způsobuje varroóza velmi časté zimní úhyny včelstev a kontrola roztoče *Varroa* se stala středem pozornosti včelařů téměř po celém světě (Rosenkranz et al., 2010).

Morfologie a biologie roztoče

Samička roztoče je 1,1-2 mm široká, hnědo-červeného zbarvení a oválného tvaru se silně sklerotizovaným dorzálním i ventrálním štítkem s větší šířkou než délkou. Nohy jsou silné a krátké, dobře přizpůsobené k pohybu na hostiteli. Sameček je výrazně menší (cca 0,7 mm), hruškovitého tvaru a světlejší barvy. Poměrem k tělu má delší končetiny než samička a je celkově méně sklerotizovaný (Rosenkranz et al., 2010; Roth et al., 2020).

Životní cyklus

Jeho životní cyklus se dělí na dvě fáze, reprodukční a disperzní (foretická). V reprodukční fázi samičky zalézají do otevřených plodových buněk krátce před jejich zavíčkováním. Preferují trubčí plod, na kterém mohou vyprodukovat 2,2-2,6 mladých samiček, zatímco na dělničích buňkách je to pouze 1,3-1,45. Dospělá samička se zpočátku ukrývá v tekuté potravě larvičky u dna buňky. Dýchání je umožněno tzv. peritreme, které funguje jako brčko a vyčnívá skrze hladinu. Po zavíčkování buňky samička roztoče vyleze na vzpřimující se larvu a propíchne jí kutikulu pro vytvoření krmícího otvoru pro sebe i budoucí potomky. To je velmi důležité pro vývoj potomků, protože sami toho nejsou schopni. Zároveň se do rány dostanou antikoagulanty, které zabraňují její zacelení (Rosenkranz et al., 2010). Vlastní reprodukci předchází krmení se tukovým tělesem larvy (Ramsey et al., 2019), protože kladení vajíček samičkou roztoče je energeticky velmi náročné a je podmíněné ziskem bílkovin, hormonů a dalších nutričních látek z tukového tělesa larvy. Některé tyto bílkoviny ani nejsou metabolizovány, ale rovnou zabudovány do struktur kladených vajíček. V principu pak vajíčka roztoče obsahují včelí bílkoviny (Tewarson, 1982; Traynor et al., 2020). Pohlaví u roztočů je determinováno arrhenotokní partenogenezí, kdy samec je haploidní se sedmi chromozomy a samice jsou diploidní se čtrnácti chromozomy (Traynor et al., 2020). Samička roztoče jako první naklade haploidní vajíčko přibližně 60-70 hodin po vniknutí do buňky. Tento první potomek je nejdůležitější, protože se z něj vylíhne sameček a musí oplodnit všechny své “sestry“, které se poté líhnou z dalších vajíček kladených jednotlivě cca každých 30 hodin. Z tohoto důvodu je první vajíčko umisťováno na horní okraj buňky, což je nejvíce bezpečné místo vzhledem ke kuklení se larvy. Páření iniciuje pohlavně dospělá samička svými feromony. Čím je mladší, tím jsou feromony silnější a sameček je preferuje. Tím je dosaženo postupného oplozování všech

samiček až do vylíhnutí včely. Sameček má krátký život a proto může být nalezen pouze v zavíčkované plodové buňce, stejně jako vývojová samičí stadia, protonymfa a deutonymfa. Jelikož je sameček málo sklerotizovaný, může snadno zemřít vlivem pohybů vyvíjející se včelí larvy, které často brání přístupu ke krmícímu otvoru (Traynor et al., 2020). V tomto případě upouštějí buňku neoplozené samičky, které však mohou naklást haploidní vajíčko do jiné plodové buňky a posléze se spářit se svým potomkem a dále klást diploidní vajíčka (Häußermann et al., 2019). V přirozených podmínkách opustí buňku spolu s matkou jedna až dvě oplozené samičky a nastane jejich foretická fáze. Samička roztoče má 1,5-3 reprodukční cykly (Rosenkranz et al., 2010).

Disperzní (foretická) fáze začíná vyběhnutím dospělé včely z buňky, na které parazitují samičky roztoče, často skryty pod sternity včely. Během této fáze se živí na tukovém tělese, což aktivuje jejich vaječníky (Ramsey et al., 2019). Mladé samičky roztoče často mění svého hostitele a preferují mladé včely, jejichž úkolem je právě péče o včelí plod. Takto se snadno dostanou do nové plodové buňky a celý cyklus se opakuje. Tato fáze roztoče je také nezbytná pro šíření roztoče do dalších úlů, zejména pokud stávající včelstvo kolabuje (Rosenkranz et al., 2010; Roth et al., 2020).

Monitoring roztoče

V případě, že včelstvo již samo nezvládá regulovat populaci roztoče *Varroa* a hrozí jeho přemnožení, je nutný zásah včelaře. Z tohoto důvodu je monitoring roztoče ve včelstvu jednou ze základních a v dnešní době nezbytných zootechnických postupů (Ramzi et al., 2017). Nejjednodušší, ale také nejpracnější metoda spočívá v mechanickém odvíčkování včelího plodu a kontrole přítomnosti roztoče. Kontrolován je přednostně trubčí plod z důvodu jeho preference roztoči. Pro přesnější informaci je však nutné ověřit větší množství plodu, který zásah nepřežije (Dietemann et al., 2013; Fries et al., 1991). Vzhledem k nerovnoměrnému napadení plodových buněk je tato metoda méně spolehlivá a neurčí celkové napadení včelstva roztočem (Roth et al., 2020). Nejčastěji se k monitoringu roztoče využívá monitorovací podložka na dně úlu, kam padají mrtví roztoči. Z důvodu odklízení nečistot z úlového dna včelami je vhodnější celozasítované dno úlu, kterým roztoči propadávají na monitorovací podložku umístěnou pod sítem. Takto se k nim včely již nedostanou. Problém však mohou způsobovat mravenci odnášející roztoče

z podložky. V těchto případech je doporučováno např. umístit nohy stojanu pro úly do nádob s olejem, kterou mravenci nejsou schopni překonat, nebo zajistit lepivý povrch na podložce. Z přirozeného denního spadu lze orientačně vyčíst celkový stav roztočů ve včelstvu. Monitorovací podložky se také využívají při hodnocení efektivity provedených ošetření, a to na základě léčebného spadu roztočů (Branco et al., 2006; Gregorc and Sampson, 2019; Peck, 2021; Shakib and Mehdi, 2017). Pro přesnější monitoring populace roztoče se používají metody využívající smyvu foretických roztočů ze vzorku včel (cca 300) odebraných z plodových plástů. Tyto včely se nasypou do upravené nádobky se sítem, posypou moučkovým cukrem a řádně protřepou, aby se obalily v cukru. Tím dojde k nastartování groomingu, mávání křídel a celkově vyšší aktivitě, která vede k zahřátí včel. Velká část roztočů je chráněná mezi sternity včel, ale zvýšená teplota je nutí vylézt a kluzká vrstva moučkového cukru na povrchu včel spolu s jejich zvýšenou aktivitou jím značně ztěžuje udržení se na včele. Po krátké době se s nádobkou opět zatřese nad bílou podložkou a spočítají se spadaní roztoči. Tato metoda má i svá omezení, např. nesmí se používat za vlhka nebo na mokré včely. Přesnost je pouze orientační, protože nikdy nespadnou všichni roztoči a záleží i na velikosti vzorku včel a místa, odkud byl odebírán (Aliano and Ellis, 2005; Fakhimzadeh, 2001a, 2001b; Gregorc et al., 2017). Další možností monitoringu je přidat do nádobky ke včelám místo moučkového cukru alkohol. Tímto se získá přesnější výsledek, ale zabije to i všechny včely (Toufailia et al., 2014). Kromě těchto možností se využívá i narkotizace CO_2 nebo N_2O. Tyto metody jsou založeny na spadu narkotizovaných roztočů ze včel po jejich protřepání. Včely se po čase proberou a mohou se vrátit zpět do úlu (Dietemann et al., 2013; Shakib and Mehdi, 2017). V případě diagnózy infestace roztoče větší než 3-5% je doporučeno okamžité ošetření včelstva (Gregorc and Sampson, 2019). Bez vhodného ošetření většina infestovaných včelstev uhyne během 1-3 let (Dietemann et al., 2013; Peck, 2021; Tihelka, 2018).

Kontrola roztoče

Syntetické akaricidy

Ke kontrole roztoče *V. destructor* se nejčastěji používají syntetické akaricidy, zejména na bázi organofosfátu kumafos, pyretroidů tau-fluvalinate, flumethrin, acrinathrin a formamidinu amitraz. Jejich výhodou je snadná aplikace, příznivá cena a vysoká účinnost v porovnání s ostatními metodami (Rosenkranz et al., 2010;

Tihelka, 2018). Tyto látky jsou však v určité míře toxické i pro včely a je proto nutné používat nejnižší možnou koncentraci, která zahubí pouze roztoče. Problém ale nastává i v případě použití nižší koncentrace, kterou jsou roztoči schopni přežít a vytvořit si určitou odolnost (rezistenci) i vůči vyšším dávkám. Z tohoto důvodu je velice důležité dodržovat přesný postup podle příbalového letáku každého léčivého přípravku. Snaha vylepšit účinnost nebo aplikační formu produktu pak vede k rychlému nárůstu rezistence roztočů na tyto látky (Gregorc and Sampson, 2019). Jelikož je většina těchto akaricidů lipofilní povahy, akumulují se jejich rezidua v malých dávkách ve včelím vosku, což způsobuje i přirozenou rezistenci roztočů, působí chronicky na dospělé včely i včelí plod a v neposlední řadě také znehodnocuje včelí produkty. Rezistence roztočů již byla pozorována na všechny výše uvedené syntetické akaricidy (Gonzalez-Cabrera et al., 2016; Hernández-Rodríguez et al., 2021; Kamler et al., 2016; Rinkevich, 2020; Stara et al., 2019). Z tohoto důvodu zůstává vývoj nových, vysoce specifických syntetických akaricidů s odlišným mechanismem účinku velkou výzvou a jejich výroba a využití je v nejbližší době nepravděpodobné (Ramzi et al., 2017). I v případě účinného ošetření může přežít malá část populace roztoče, která se poté nekontrolovaně rozmnoží a vytvoří rezistentní populaci. Střídání různých účinných látek se toto riziko minimalizuje a na většině míst zůstává jediným využitelným řešením. I tato metoda je však pouze dočasná z důvodu vzniku rezistence na více účinných látek najednou. Vznik multirezistence je velmi usnadňován přenosem roztočů včelami mezi jednotlivými úly i stanovišti (Ramzi et al., 2017; Rosenkranz et al., 2010).

Míra negativního účinku syntetických pesticidů na včely (review v Tihelka, (2018) závisí na mnoha faktorech, např. na metodě aplikace, koncentraci a dávce pesticidu, nebo přítomnosti dalších stresových faktorů, mezi které patří klimatické faktory, omezená pylová snůška, kočování se včelstvy, nebo patogenní zátěž. Většinou se tento negativní efekt projevuje zvýšenou mortalitou včel a zkrácením jejich života (Pettis et al., 1991; Tihelka, 2018). Objevují se však i změny v chování včel, např. redukovaná míra trofolaxe a v důsledku toho ovlivnění energetické distribuce v celé kolonii (Bevk et al., 2012), nebo změny v metabolismu vedoucí ke snížení množství proteinů, sacharidů, tuků i enzymů v hemolymfě (Loucif-Ayad et al., 2010, 2008). Včelí matky mohou být syntetickými pesticidy také negativně ovlivněny, a to nejen jejich reprodukční vlastnosti jako je nižší hmotnost vaječníků nebo nižší počet uchovávaných spermií i produkovaných vajíček, ale i chování

matky a zkrácení života (Collins and Pettis, 2013; Pettis et al., 2004; Rangel and Tarpy, 2015). U trubců bylo pozorováno snížení celkové hmotnosti, menší počet spermií a jejich nižší životnost. To může vést k nedostatečnému oplození matky a nutnosti její dřívější výměny (Burley et al., 2008; Johnson et al., 2013). Značně je ovlivňován i včelí plod, protože je neustále v kontaktu s rezidui pesticidů ve voskových buňkách (Orantes-Bermejo et al., 2010). Byla prokázána jeho vyšší mortalita, kratší délka života v dospělosti a zpožděný larvální vývoj, který zároveň prodlužuje reprodukční cyklus roztoče *Varroa* v plodových buňkách a vede k produkci více samiček (Medici et al., 2012; Wu et al., 2011). U všech kast pak může docházet k negativnímu efektu ovlivňujícímu učení a paměť včel (Gashout et al., 2020), expresi genů zodpovědných za imunitu a detoxifikaci (Gashout et al., 2018) a také k poškození čichových senzorů, které jsou klíčové pro nezbytnou komunikaci a získávání potravy (Weick and Thorn, 2002). V neposlední řadě chronické působení reziduí pesticidů zvyšuje náchylnost včel k houbovým (Wu et al., 2012) a virovým onemocněním (Locke et al., 2012). Směs akaricidů může navíc způsobit tzv. koktejlový efekt, kdy jejich směs působí synergicky (Johnson et al., 2009; Traynor et al., 2016), popřípadě chronicky a poškozuje tak včely mnohem více (de Mattos et al., 2017).

Tyto syntetické pesticidy mohou také negativně působit i na lidské zdraví. Vzhledem k jejich schopnosti bioakumulace se hromadí v těle a působí chronicky. Často jsou spojovány s potlačováním imunity, narušením hormonální aktivity, nervovými poruchami, genetickými změnami a vznikem rakoviny (Al-Waili et al., 2012). Proto je nezbytné zapojit do kontroly varroózy alternativní metody (Ruffinengo et al., 2014).

Kontrola roztoče pomocí syntetických akaricidů je v oblibě zejména na velkých farmách v USA, zatímco drobnochovatelé používají více alternativní metody nebo nechávají včelstva bez ošetření. V poslední době však i tito drobnochovatelé začínají používat více syntetických akaricidů, pravděpodobně vzhledem k zvyšujícím se úhynům včelstev (Haber et al., 2019). V České republice je trend opačný a po zrušené povinnosti léčit syntetickými akaricidy mnoho včelařů přechází k alternativním metodám, které jsou běžnou praxí i v Rakousku (Brodschneider et al., 2019).

Organické kyseliny

Další možností kontroly roztoče *Varroa* je použití přípravků na bázi organických kyselin. Nejčastěji se využívá kyselina mravenčí, šťavelová a mléčná. Jejich výhoda spočívá v dostatečné efektivitě při správném použití, minimálnímu riziku vzniku rezistence roztočů a malému riziku akumulace reziduí, protože většina těchto látek je rozpustná ve vodě, těkavá anebo představují přirozenou složku medu. Tyto přípravky mají však i své nevýhody, jako je např. aplikační forma nebo dávka/koncentrace účinné látky. Aplikačních forem je několik, včetně odparu, pokapu, postřiku, fumigace nebo krmení v roztoku. Každá z nich má různou účinnost v závislosti na klimatických podmínkách i povaze aplikované látky. Dalším problémem je stanovení léčebné dávky tak, aby byla účinná a zároveň nepoškozovala včely, protože tyto látky mají většinou malé rozpětí mezi toxicitou k roztočům a toxicitou ke včelám, zejména včelímu plodu. Každé včelstvo je různě silné, obsedá různě velký prostor a bývá problém zvolit správnou dávku léčiva. Značný vliv mají i klimatické podmínky nebo konstrukce úlů. Dalším omezením je použití pouze v bezplodovém období pro zachování deklarované účinnosti. Výjimkou je kyselina mravenčí, která prostupuje i do zavíčkované buňky. Z těchto důvodů je aplikace složitější než u syntetických akaricidů a vyžaduje určitou znalost biologie a reprodukce roztoče *Varroa* (Rosenkranz et al., 2010).

Kyselina šťavelová působí na roztoče dvojím způsobem. Kromě kontaktní toxicity, která poškozuje zejména ústní ústrojí, zvyšuje také kyselina šťavelová aktivitu včel a tím podporuje grooming (Al Toufailia et al., 2015). Kyselina šťavelová se nejčastěji aplikuje pokapem roztoku dihydrátu kyseliny šťavelové v sacharózovém roztoku v různém poměru. Do každé uličky obsazené včelami se injekční stříkačkou aplikuje 5 ml roztoku (Al Toufailia et al., 2015; Gregorc et al., 2017; Papežíková et al., 2017). V případě použití pokapu kyselinou šťavelovou ve třech ošetření v bezplodovém období bylo dosaženo vysoké účinnosti (99%), v přítomnosti včelího plodu však účinnost výrazně klesá (39 a 52%) (Gregorc and Planinc, 2001). V teplejších podmínkách, kdy včely nezimují v chomáči, ale jsou spíše rozvolněné, bylo stejnou metodou ve čtyřech ošetřeních v zimním období dosaženo účinnosti 90-100%. V letních měsících však byla účinnost minimální (21%) (Gregorc et al., 2017). Další způsoby aplikace jsou postřikem na jednotlivé rámky, nebo sublimací. Tyto dva postupy se jeví v porovnání v zimním bezplodovém období jako účinnější, zejména sublimace, u které pro zachování vysoké účinnosti

stačí nižší dávka než v případě ostatních způsobů aplikace (Al Toufailia et al., 2015). Pro včely se sublimace jeví jako bezpečné ošetření (Al Toufailia et al., 2015; Papežíková et al., 2017) na rozdíl od pokapu, postřiku, nebo orální aplikace, jež vykazují kontroverzní výsledky (Ebert et al., 2007; Gregorc and Škerl, 2007; Hernández et al., 2007; Schneider et al., 2012; Toomemaa et al., 2010). Dávka kyseliny šťavelové využívaná v praxi je však nižší než ve výše uvedených studiích, cca 70 ug na včelu (Charriére and Imdorf, 2002), což je mnohem méně než stanovená LD_{10} (176,68 ug) (Aliano et al., 2006). Zajímavé výsledky přináší nový typ aplikace rozpuštění dihydrátu kyseliny šťavelové v glycerinu aplikované na celulózový nosič. Takto aplikovaná kyselina šťavelová má dlouhodobý účinek (až 42 dní) a proto i vysokou účinnost za přítomnosti plodu (93,1%) (Maggi et al., 2016). Jiná studie však ukazuje účinnost nižší (78,7%) a zároveň vyšší mortalitu včel (Sabahi et al., 2020).

Kyselina mravenčí je výjimečná tím, že působí jak na roztoče ve foretické fázi, tak i na roztoče reprodukující se v zavíčkovaných buňkách (Pietropaoli and Formato, 2019; Rosenkranz et al., 2010). Používá se v různých aplikátorech, jejichž hlavním cílem je její rovnoměrné uvolňování. Za vysokých teplot se přirozeně odpařuje velmi rychle a působí toxicky i na včely, včelí plod a zejména na vajíčka (Giusti et al., 2017). Naopak za chladných dní se odpařuje velmi pomalu a nedosahuje požadovaného efektu na roztoče. Ideální venkovní teplota pro účinné ošetření kyselinou mravenčí je 15 – 30 °C. Jednou z možností jsou gelové nosiče (Varterminator, MAQS), které se umisťují nad plodové rámky. Druhou možnost představují celulózové nosiče, které jsou nasyceny kyselinou a dochází k jejímu odparu. Při vhodném použití mají obě formy aplikací vysokou účinnost (57-99%) (Giusti et al., 2017; Pietropaoli and Formato, 2019, 2018; Satta et al., 2005).

Kyselina mléčná se používá nejčastěji postřikem 15% vodního roztoku na rámky obsednuté včelami. Z každé strany se aplikuje 5ml. Použití kyseliny mléčné je vhodné v období bez plodu. Za těchto podmínek bylo dosaženo vysoké účinnosti (88-90%). Negativní vliv na rozvoj včelstva nebyl zaznamenán (Domatskaya and Domatsky, 2020). Tato metoda je však vhodná spíše pro malá včelstva, jako jsou oddělky, smetence, nebo roje, protože je více časově náročná (Rosenkranz et al., 2010). Avšak za přítomnosti plodu ve včelstvu v přímém porovnání těchto tří organických kyselin byla kyselina mléčná nejméně účinná, a to jak na podzim (54-88%) tak i na jaře (8%) (Girişgin and Aydin, 2010).

Mechanické metody kontroly

V poslední době jsou také hojně využívané mechanické metody kontroly. Mezi ně patří tzv. trapping comb method, kde se využívá akumulace roztočů v zavíčkovaných plodových buňkách včel, především trubčích. Dále je několik možností, jak s tímto plodem nakládat. Trubčí plod se může buď odstranit (usmrtit) (Calderone, 2005), umístit do jiného včelstva, které se po jejich vylíhnutí ošetří syntetickými akaricidy (Wantuch and Tarpy, 2009), případně ještě zavíčkovaný plod ošetřit kyselinou mravenčí, což zamezí dalšímu šíření roztočů (Pietropaoli and Formato, 2019, 2018). Také je možné využití trubčího plodu v lidské výživě jako zdroje kvalitních bílkovin (Lecocq et al., 2018; Ulmer et al., 2020), nebo vzhledem k vysokému obsahu bioaktivních látek (Silici, 2019) lze také využít ve formě homogenátu ve zdravotnictví pro léčbu různých nemocí a obtíží spojovaných zejména s neplodností (Sidor and Dżugan, 2020). V zemědělství je možné využití homogenátu jako podpůrného prostředku pro hospodářská zvířata (Bolatovna et al., 2015). Jiná možnost je termické ošetření při teplotě 41-44°C po dobu 2-3 hodin ve specializovaných boxech, a to jak dělničího, tak i trubčího plodu. Při teplotě 42-43,7°C bylo dosaženo vysoké účinnosti kontroly roztoče *Varroa* a dokonce i prodloužení délky života vylíhnutých včel z takto ošetřeného plodu (Kablau et al., 2020, 2019). Avšak kromě vysoké počáteční investice do termického boxu a větším časovým nárokům na ošetření patří mezi další nevýhody riziko usmrcení části plodu (Kablau et al., 2019).

Jiná metoda využívá posypu včel práškovým cukrem. Zajímavé výsledky ukázaly laboratorní pokusy (Aliano and Ellis, 2005; Fakhimzadeh, 2001a), ale v praxi je účinnost nízká (Berry et al., 2012; A. M. Ellis et al., 2009; Haber et al., 2019). Neúčinný se zdá být i chov včel na menších buňkách (4,9 mm) v porovnání s klasickým rozměrem (5,3 mm), které měly omezit prostor v buňce pro vývoj roztoče, případně urychlit vývoj včely (Berry et al., 2010; A. Ellis et al., 2009; Seeley and Griffin, 2011).

Genetické metody kontroly

Mezi genetické metody patří šlechtění na *Varroa* senzitivní hygienu (VSH). Ukazuje se, že je možné dále šlechtit včelstva, která přežila plošné kolapsy způsobené varroózou. Tato včelstva se lépe vypořádají s roztoči odstraňováním

napadeného plodu nebo groomingem. I přes mnohé úspěchy však tato metoda kontroly roztoče ve většině případů stále není dostatečná a i tato včelstva je nutné dříve nebo později ošetřit (Kirrane et al., 2018; Ward et al., 2008). Navíc se ukazuje, že včelstva šlechtěná na VSH mohou mít i nižší produkci medu (Le Conte et al., 2007).

Další oblastí intenzivního výzkumu je využití RNA interference (RNAi) k regulaci cílových genů roztoče *Varroa*. Tato metoda využívá dvouvláknovou RNA (dsRNA), která je komplementární k cílovému úseku mediátorové RNA (mRNA). Po vniknutí molekul dsRNA do organismu dojde k jejich rozštěpení na menší úseky, tzv. siRNA, o délce 19 – 25 nukleotidů, které se naváží na komplementární mRNA a tím je degradují, čímž zabrání genové expresi (Fire et al., 1998). Na stejném principu funguje i přirozená obrana včel proti virům (Flenniken and Andino, 2013). Většinou je záměrem přerušení syntézy důležitých proteinů cílového organismu, což vede k jeho úhynu. Pomocí této metody bylo dosaženo několika úspěšných pokusů redukujících počty roztočů, a to jak v *in vitro* podmínkách (Campbell et al., 2016; Huang et al., 2019), tak i v prostředí úlu (Garbian et al., 2012; Leonard et al., 2020). Částice dsRNA však podléhají poměrně rychlému rozkladu, proto je důležité jejich kontinuální podávání. Způsob jejich doručení do cílového místa se zachováním dostatečné účinnosti je značně problematický a závisí na vývojovém stadiu jedince, stabilitě cílového genu, místě cílové tkáně a dostatečné kvantitě dsRNA (Yang et al., 2018). Tyto látky se aplikují do organismu včel, odkud se dále šíří do roztočů. Jednou z možností jak tyto molekuly genetické informace dostat do organismu včely v dostatečném množství je např. krmením cukerným roztokem (Garbian et al., 2012), nebo genetickou modifikací symbiontních bakterií zajišťujících produkci těchto specifických látek (Leonard et al., 2020). Tato metoda je však pro využití v praxi velmi nákladná a může ovlivňovat i necílové úseky genetické informace. Proto je z hlediska bezpečnosti nezbytné, aby tyto dsRNA úseky byly navrženy tak, aby se neshodovaly s jinými necílovými úseky RNA včely, člověka, ani dalších organismů. To je zatím velmi problematické a zejména u savců, ale i u včel, způsobuje cizorodá dsRNA nespecifické změny genové exprese (Jarosch and Moritz, 2012; Nunes et al., 2013). Dalším omezením může být dlouhodobou koevolucí vyvinutý obranný mechanismus některých virů, který způsobuje produkci specifických látek eliminujících dsRNA (Chen et al., 2014).

K úpravě genetické informace lze použít také metodu CRISPR – Cas9. Jde o cílenou editaci genomu přímo na molekule DNA. Takto lze vypnout celé geny, případně upravit jejich funkci. Na rozdíl od RNAi je tato metoda trvalá (Taning et al., 2017). Na včelách již byla úspěšně aplikována při modifikaci genu odpovědného za produkci MRJP1 proteinu (Kohno et al., 2016), nebo při snaze ovlivnit vývoj vaječníků a tím diferenciaci kast mezi matkou a dělnicí (Roth et al., 2019). V Evropské unii je však tato metoda omezena na využití pouze v laboratořích, protože pro praktické využití je stejně jako GMO legislativně regulováno (Taning et al., 2017).

Biologické metody kontroly

Výzkum probíhá i v oblasti přirozených antagonistů roztoče *Varroa*, jako jsou např. viry (Zhang et al., 2007), bakterie (Saccà and Lodesani, 2020), draví pavoukovci (Rangel and Ward, 2018; Read et al., 2014) a entomopatogenní houby (Meikle et al., 2012). Největší úspěch byl zaznamenán u entomopatogenních hub, konkrétně *Metarhizium anisopliae* (Kanga et al., 2003), *Beauveria bassiana* (García-Fernández et al., 2008; Meikle et al., 2008) nebo *Verticillium lecanii* (Shaw et al., 2002). Zatím se však tento způsob kontroly roztoče *Varroa* do běžné praxe nedostal, protože vykazuje většinou spíše průměrnou nebo nižší účinnost (Romo-Chacón et al., 2016; Sinia and Guzman-Novoa, 2018).

Největší potenciál v současné době mají biopesticidy na bázi rostlinných silic. Tyto přírodní látky se zdají být vhodnou alternativou k syntetickým akaricidům a mohly by tak představovat základní složku integrované ochrany proti parazitům (Pavela and Benelli, 2016; Perricone et al., 2015). Mnoho rostlinných druhů bylo využíváno v medicíně dlouho před objevením mikrobů. Naši předci empiricky pozorovali léčivé účinky některých rostlinných druhů, dnes označované jako antimikrobiální efekt. Za tento efekt jsou zodpovědné rostlinné oleje obsahující těkavé substance různých bioaktivních látek. Mají silné aroma a v rostlině zastávají ochranné funkce. Tyto rostlinné oleje jsou hojně využívané i v dnešní době k prevenci a léčbě různých zažívacích a respiračních onemocnění, převážně v podobě čajů, koření nebo tradičního léčitelství. Navíc nemají žádné, nebo pouze minimum vedlejších účinků. Z těchto důvodů jsou rostlinné oleje považovány jako ekologicky šetrné a bezpečné i v lidské výživě (GRAS status). I v současné době jsou stále

účinné, jelikož nebyla zaznamenána žádná rezistence patogenů nebo parazitů (Bakkali et al., 2008; Kuzyšinová et al., 2016; Li et al., 2019).

Rostlinné oleje obsahují obecně řadu různých chemických látek, zejména fenoly, terpeny a terpenoidy, z nichž některé mohou působit synergicky a mají velmi široké spektrum účinnosti (Li et al., 2019; Ramzi et al., 2017; Rios and Recio, 2005). Kromě hlavních komponent obsahují rostlinné oleje velké množství dalších látek v nízkých koncentracích, které usnadňují penetraci buněk a fixaci na buněčné membrány, čímž zvyšují celkovou účinnost (Tak and Isman, 2017). Z tohoto důvodu je vznik rezistence u patogenů velmi nepravděpodobný. Tyto látky vznikají v mnoha aromatických rostlinách jako sekundární metabolity, kde slouží primárně jako antimikrobiální ochrana (Bakkali et al., 2008). Kromě toho se významně podílejí na lákání opylovatelů, a tím i zajištění pohlavní reprodukce rostlin (Stevenson, 2020). Pro komerční využití jsou rostlinné oleje získávány destilací, lisováním, nebo extrakcí různých částí aromatických rostlin, zahrnující listy, stonky, větvičky, semena, plody, květy, pupeny, kořeny, dřevo, nebo kůru. Výsledkem je koncentrovaný roztok těkavých organických komponent charakteristických silnou vůní (Ferrentino et al., 2020).

Jako přírodní a snadno odbouratelné produkty nezanechávají rostlinné oleje žádná, nebo pouze minimální rezidua, které nepřekračují doporučené limity a velmi rychle se odbourají kompletně (Milano and Donnarumma, 2017; Serra Bonvehí et al., 2016). Mohou tak být využívány i v ekologickém zemědělství. Vyznačují se kontaktní toxicitou, repelentním účinkem, inhibují reprodukci a příjem potravy (Bakkali et al., 2008; Regnault-Roger et al., 2012) a v některých případech mohou u včel stimulovat hygienické chování a grooming (Abd El-Wahab et al., 2012). Fenolické látky obsažené v rostlinných olejích také zvyšují detoxikační schopnost včel a snižují jejich mortalitu při intoxikacích pesticidy (Hýbl et al., 2021b).

Mnoho studií dokazuje inhibiční účinek rostlinných olejů na včelí patogeny a parazity, zejména na *A. apis* (Ansari et al., 2017; Calderone et al., 1994; Chaimanee et al., 2017; Chantawannakul et al., 2003; Gabriel et al., 2018), *P. larvae* (Calderone et al., 1994; Chaimanee et al., 2017; Damiani et al., 2014), *V. destructor* (Ariana et al., 2002; Conti et al., 2020; Damiani et al., 2009; Ramzi et al., 2017) a *N. ceranae* (Borges et al., 2020; Bravo et al., 2017).

Účinnost rostlinných olejů je mnohem vyšší než účinnost přirozených antagonistů *Varroa* (Romo-Chacón et al., 2016; Sinia and Guzman-Novoa, 2018),

v některých případech i na úrovni syntetických akaricidů (Ramzi et al., 2017). Naopak toxicita pro včely je nízká, dokonce i v porovnání s hojně využívanými organickými kyselinami (Ebert et al., 2007; Sabahi et al., 2020).

Vysokou účinnost vykazuje olej z rostlin rodu dobromysl (*Origanum*), jejichž hlavní složku tvoří carvacrol (Ariana et al., 2002). V kombinaci s tymiánovým (*Thymus satureioides*) olejem chemotypu borneol má srovnatelnou účinnost se syntetickými akaricidy (flumethrin). Tato kombinace naznačuje synergický účinek mezi carvacrolem a borneolem (Ramzi et al., 2017). Další synergický efekt byl nalezen mezi oleji z máty, merlíku a vavřínu (Aglagane et al., 2021). Kombinacemi vhodných olejů tak lze dosáhnout vysoké účinnosti i při nižší dávce.

Jednou z nejvíce účinných látek rostlinných olejů je monoterpen tymol, který se přirozeně vyskytuje v tymiánovém oleji a v praxi se již využívá v několika přípravcích (Sabahi et al., 2020). Na roztoče působí neurotoxicky (Rattan, 2010). Jeho účinnost je jako v případě všech rostlinných olejů závislá na aplikační formě a venkovní teplotě. Při správném použití je velmi vysoká, a to jak ve formě prášku (96,6%), tak i v kombinaci s glycerinem (92,4%) (Sabahi et al., 2020). Poněkud horších výsledků bylo dosaženo aplikací tymolu ve formě gelu nebo celulózových nosičů (Gregorc and Planinc, 2012). Jeho účinnost je možné navýšit v kombinaci s mechanickými metodami na více než 98% (Cengiz, 2018). Tymol má také pozitivní účinky na hygienické chování včel. Po jeho aplikaci byla prokázána zvýšená míra odstraňování poškozeného plodu (Colin et al., 2019).

Z rostlinných olejů a jejich komponent však patří tymol i mezi nejvíce toxické vůči včelám. Selekční poměr (selectivity ratio) mezi LC_{50} na včelu (3,3 µg) a LC_{50} na roztoče (2,6 µg) po 48 hodinách je velmi úzký a indikuje možné problémy při nepřesné aplikaci (Brasesco et al., 2017). Rostlinný olej z tymiánu má toto rozmezí širší (LC_{50}=11,9 µl, 3,02 µl), ale stále patří k nejvíce toxickým olejům vůči včelám (Damiani et al., 2009). Použití tymolu je tedy omezené a za vysokých venkovních teplot (27°C) může vykazovat zvýšenou mortalitu včel (Gal et al., 1992). I v nízké koncentraci (0,05%) způsobuje zvýšení hladiny enzymů spojených s neurotransmisí a detoxikací indikující zvýšenou potřebu detoxikace a chronický dopad na nervovou soustavu (Glavan et al., 2020).

Zajímavých výsledků bylo dosaženo i s dalšími rostlinnými oleji. Například LC_{50} levandulového oleje (*Lavandula officinalis*) byla po 48 hodinách (3,58 µl) srovnatelná s tymiánovým olejem a po 72 hodinách dokonce ještě účinnější (2,24 µl).

Ke včelám je mnohem šetrnější (LC_{50}=>20 µl). Hlavní složkou levandulového oleje je lavandin (Damiani et al., 2009). Další *in vitro* testy ukazují vysokou účinnost, ale pouze bez přítomnosti včel (Ariana et al., 2002). Vysokou účinnost má také olej z rodu saturejka *(Satureja*), a to i při nízké koncentraci (Ariana et al., 2002).

Problém však představuje nestálé složení biologicky aktivních látek, a to i ve stejném rostlinném druhu. Toto složení je ovlivněno částí rostliny, ze které byl olej získán, klimatickými podmínkami, půdními charakteristikami, věkem a vegetačním cyklem rostlin a jejich genetickými znaky. Kolísavé složení účinných látek ze stejného rostlinného druhu způsobuje značné rozdíly v účinnosti a komplikuje využití rostlinných olejů v praxi (Angioni et al., 2006; Masotti et al., 2003; Ramzi et al., 2017; Tock et al., 2020).

2.4.2 Zvápenatění včelího plodu

Zvápenatění včelího plodu patří mezi nejvíce rozšířené onemocnění včel. Toto onemocnění je vyvoláno heterotalickým houbovým patogenem *Ascosphaera apis* (Maassen ex Claussen) (Spiltoir, 1955), který napadá pouze včelí plod. Dospělé včely se nakazit nemohou, pomáhají však s rozšiřováním onemocnění přenosem spor, tzv. askospor (Ansari et al., 2017). Na rozdíl od dalších entomopatogenních hub řádu Hypocreales však tyto askospory nemohou klíčit na povrchu hostitele a musí být přijímány orálně (Mannino et al., 2019). Jakmile se askospory dostanou do zažívacího traktu včelí larvy spolu s přirozenou potravou, rychle se aktivují vystavením vyšší koncentrace CO_2 a začnou klíčit (Heath and Gaze, 1987). Následně prorazí peritrofickou membránu a prorostou celou larvou. Zpočátku infekce nakažená larva přestane přijímat potravu, později opuchne a na jejím povrchu se vytvoří husté bílé mycelium patogena. Nakonec se larva scvrkne do podoby připomínající šedou až černou nebo bílou křídu, v závislosti na přítomnosti reprodukčních struktur houby (Aronstein and Murray, 2010). V případě přítomnosti obou pohlavních typů začne patogen produkovat velmi odolné spore cysts (ascomata), ve kterých jsou chráněné nové askospory v kulovitých útvarech zvaných spore balls. Tyto spory jsou životaschopné po mnoho let (Bamford and Heath, 1989). Takto usmrcené larvy včel se označují jako zvápenatělé mumie (chalkbrood mummies) a představují typické příznaky tohoto onemocnění (Aronstein and Murray, 2010). Zvápenatění včelího plodu je považováno za onemocnění vyvolané stresem a vyskytuje se převážně ve vlhkých a chladnějších klimatických

podmínkách, nebo v úlech slabších včelstev, která nejsou schopna udržet optimální stálou teplotu 35°C pro chov plodu (Evison, 2015). Houba *A. apis* za této teploty roste velmi pomalu a nepravidelně a preferuje raději teploty kolem 30°C (Mráz et al., 2021b). To je také jeden z důvodů, proč larvy stresované chladem jsou častěji napadeny tímto patogenem (Vojvodic et al., 2011). Vhodnými teplotními podmínkami a vlivem výživy na tohoto patogena se zabývá Mráz et al. (2021).

Celosvětově je výskyt tohoto onemocnění na vzestupu (Aronstein and Murray, 2010), zejména pak v jihovýchodní Asii (Chantawannakul et al., 2003; Li et al., 2018), kde způsobuje velké škody. Ve většině případů však nezpůsobuje úhyny celých včelstev, ale podílí se na jejich oslabení a snížení produktivity (Zaghloul et al., 2005). Navíc snižuje jejich imunitu, čímž se stávají více náchylná k dalším patogenům i kontaminantům životního prostředí (Hedtke et al., 2011).

Vzhledem k absenci terapeutického přípravku určeného k léčbě zvápenatění včelího plodu a zákazu používání fungicidů k těmto účelům v mnoha státech, včetně EU, je kontrola *A. apis* velmi obtížná (Guo et al., 2018). Používají se zejména preventivní opatření jako je chov odolnějších včelstev (Spivak and Reuter, 2001) nebo je kladen důraz na sanitaci a správnou včelařskou praxi (Flores et al., 2005). Z těchto důvodů je velký zájem o alternativní metody kontroly (Ansari et al., 2017), z nichž největších úspěchu bylo dosaženo pomocí rostlinných silic, např. tymiánové, cedrové, hřebíčkové a skořicové (Mraz et al., 2019).

2.4.3 Mor včelího plodu (MVP)

Mor včelího plodu je onemocnění způsobené gram pozitivní tyčinkovitou bakterií *Paenibacillus larvae* (White, 1906). Jedná se o fakultativně anaerobní sporulující bakterií dorůstající délky 2,5-5 μm. Spory o velikosti 0,-1,3 μm jsou velice odolné a mohou přežívat i více než 35 let. Zároveň jsou jedinou infekční formou bakterie a mohou infikovat pouze včelí plod (Hansen and Brødsgaard, 1999; Hitchcock et al., 1979; Neuendorf et al., 2004). Nejvíce náchylné jsou larvy ve stáří 12-36 hodin od vylíhnutí, které se snadno infikují stravou obsahující i malé množství spor (Genersch et al., 2005). Starší larvy získávají stále větší odolnost, která je dána také geneticky a dospělci se již nakazit nemohou. Vzhledem k velké odolnosti spor a jejich schopnosti akumulovat se a přežívat v těle dospělců však tito jedinci často slouží jako přenašeči onemocnění (Poppinga and Genersch, 2015).

Patogeneze

Spory ve střevě larvy začnou velmi rychle klíčit, čímž se utvoří vegetativní forma bakterie schopná pohybu a rozmnožování. Tyto bakterie pravděpodobně využívají přijímanou potravu od larvy, protože obsahují enzymy podílející se na štěpení některých sacharidů. To umožňuje rychlý růst a rozvoj vedoucí k zaplnění velké části střeva larvy (Genersch, 2010b; Poppinga and Genersch, 2015) bez významného poškození epitelu (Yue et al., 2008). Následně dochází k průniku bakterií přes peritrofickou membránu za pomoci aktivních extracelulárních proteáz (Antúnez et al., 2009) a mechanického pohybu bakterií (Salyers and Whitt, 2002), čímž dojde k úmrtí larvy (Yue et al., 2008). Proteázy slouží také k celkovému rozkladu larvy na tzv. příškvar, což je typický příznak onemocnění. Na rozdíl od bakterie *Melissococcus plutonius*, způsobující hnilobu včelího plodu, lze tento příškvar odstranit jen velmi obtížně. Buňky s infikovaným plodem jsou ztmavlé, propadlé a někdy proděravělé po uniklém plynu, případně může dojít k vytlačení tekutiny na povrch víčka buňky. Dalším typickým příznakem je mezerovitost plodu, která vzniká na základě hygienického chování včel odstraňováním uhynulých larev (Hansen and Brødsgaard, 1999). *P. larvae* v rozkládající se larvě začne produkovat spory umožňující snadné šíření onemocnění. Mladé dospělé včely čistící buňky s příškvary, které obsahují až 2,5 bilionu spor, tyto spory roznášejí nejen ve své kolonii, ale i mezi ostatními koloniemi v doletu včel (Genersch, 2010b). Mor včelího plodu se šíří velmi snadno i z kontaminovaného medu, pylu, vosku a včelařského vybavení. Další riziko mohou představovat ulétlé roje. Některé studie však toto tvrzení vyvracejí s tím, že u divoce žijících včelstev se MVP téměř nevyskytuje a tudíž nepředstavuje infekční tlak (Goodwin et al., 1994; Hornitzky et al., 1996). Z těchto důvodů je onemocnění mor včelího plodu celosvětově rozšířené s výjimkou subsaharské Afriky a Indického subkontinentu (Hansen and Brødsgaard, 1999) a je považováno za jedno z nejvíce infekčních. Při jeho potvrzení platí přísná karanténní opatření spolu s pálením všech MVP pozitivních včelstev i s úly a veškerým spalitelným vybavením, což způsobuje značné ekonomické ztráty (Titěra, 2009).

Klasifikace

Onemocnění mor a hniloba včelího plodu bylo popsáno již v roce 1769 pod souhrnným názvem Foulbrood ale až v roce 1906 úspěšnou kultivací *P. larvae* (tehdy ještě *Bacillus larvae*) došlo k rozdělení těchto onemocnění, jak je známe nyní

(White, 1906). Později byla z příškvarů objevena podobná bakterie pojmenována *Bacillus pulvifaciens* (Katznelson, 1950). S příchodem molekulárně genetických metod došlo na základě odlišností k přeřazení těchto druhů do samostatného rodu *Paenibacillus* (Ash et al., 1993). O 3 roky později došlo k dalšímu přeřazení a vznikly dva poddruhy, *P. larvae* subs. *larvae* a *P. larvae* subs. *pulvifaciens* (Heyndrickx et al., 1996). K zatím poslední klasifikaci došlo na základě zkoumání genotypů pomocí ERIC (Enterobacterial repetitive intergenic consensus) primerů a výsledkem bylo odstranění poddruhů a přijetí 4 genotypů (ERIC I – IV) s tím, že původní poddruh *P. larvae* subs. *larvae* představuje ERIV I a II, a poddruh *P. larvae* subs. *pulvifaciens* představuje genotyp ERIC III a IV (Genersch et al., 2006).

Tab. č. 1 ERIC genotypy *Paeniballus larvae* (Genersch, 2010b)

Paenibacillus larvae				
Genotyp	ERIC I	ERIC II	ERIC III	ERIC IV
Morfologie kolonií	našedlá	oranžová	oranžová	našedlá
Poddruh	*P. l.* subsp. *larvae*	*P. l.* subsp. *larvae*	*P. l.* subsp. *pulvifaciens*	*P. l.* subsp. *pulvifaciens*
LT_{100}	12 d	7 d	7 d	7 d

Podle tabulky je zřejmé, že na úrovni jedince jsou více virulentní genotypy ERIC II-IV, jelikož usmrtí larvu přibližně za 7 dní, na rozdíl od genotypu ERIC I, který k tomu potřebuje průměrně 12 dní (Genersch et al., 2006). Na úrovni kolonie je však virulence opačná, protože larvy uhynulé ještě před zavíčkováním jsou snadno rozpoznány a odstraňovány včelami dříve, než bakterie vysporulují. Tím se významně snižuje infekční tlak a zpomaluje průběh onemocnění. Naopak larvy uhynulé v již zavíčkované buňce jsou pro včely obtížnější detekovat, čímž dochází k častější tvorbě příškvarů plných infekčních spor (Evans and Spivak, 2010; Rauch et al., 2009).

I přes vysokou infekčnost onemocnění zůstávají některá včelstva v ohniscích nákazy MVP prostá, nebo bez klinických příznaků (Hansen and Brødsgaard, 1999). Tyto rozdíly jsou pravděpodobně způsobeny různou tolerancí včelstev a jejich úrovní hygienického chování. Velmi významný faktor je i virulence kmene *P. larvae* nebo napadení včelstva jinými patogeny nebo parazity, zejména kleštíkem včelím. Proto nelze jednoznačně určit, jaké množství spor je nutné k vypuknutí onemocnění. V některých případech je i obtížné detekovat klinické příznaky, zejména

v souvislosti rychlého odstraňování uhynulých larev před vznikem příškvarů (Genersch, 2010b).

Prevence a kontrola MVP

Nejdůležitější opatření pro předcházení onemocnění je prevence, spočívající v kontrole plodových rámků, časté obměně včelího díla, dezinfekci nástavků a chovu silných a odolných včelstev (Titěra, 2009). Velkým rizikem je krmení včel medem nebo pylem neznámého původu. Existují však i metody, které pomáhají odhalit původce onemocnění o několik let dříve, než se vyskytnou ve včelstvu klinické příznaky onemocnění, např. bakteriologické vyšetření z medu, nebo ze směsného vzorku včel (von der Ohe, 2003). V případě absence klinických příznaků u včelstev se ještě doporučuje metoda přemetení včelstev na mezistěny (Hansen and Brødsgaard, 1999; Munawar et al., 2010).

V případě potvrzení klinických příznaků dochází k propuknutí onemocnění. V některých státech (USA, Kanada, Argentina) se k potlačení MVP, ale i preventivně používají antibiotika, např. oxytetracyklin hydrochlorid (OTC) a sulfathiazol. Dlouhodobou expozicí však může vznikat rezistence. Tyto látky se navíc používají i k léčbě bakteriálních nemocí u lidí, což může v případě rezistence vést ke značným problémům. Z těchto důvodů je používání antibiotik ve včelstvech v mnoha státech zakázáno (EU). Další negativní efekt představují rezidua hromadící se ve včelích produktech, kde ovlivňují jejich kvalitu a zdravotní nezávadnost. Tyto kontaminované produkty představují další komplikace stálou expozicí vůči včelám, na které může působit chronicky, vyvolávat stres, negativně ovlivňovat včelí mikroflóru a snižovat životaschopnost včelích larev. Antibiotika však včelstva od moru včelího plodu nevyléčí, ale pouze utlumí klinické příznaky, protože nepůsobí na odolné spory. Tím se zkresluje průběh onemocnění a po vysazení antibiotik mor včelího plodu opět propukne. V České republice je jediné povolené opatření zabraňující šíření MVP zákonem nařízené spálení infikovaných včelstev (Titěra, 2009).

Vzhledem k zákazu antibiotik ve včelařství v EU je snaha o alternativní přístupy léčby a potlačování MVP. Určitého úspěchu již bylo dosaženo šlechtěním včelstev na vysokou úroveň hygienického chování dospělých včel a vyšší odolnosti včelího plodu. V principu jde o včasné nalezení a odstranění napadeného plodu, čímž se zabrání další produkci spor a sníží se infekční tlak (Rauch et al., 2009; Spivak and

Reuter, 1998). Vliv na odolnost včelího plodu mají také různé včelí kasty i kvalita stravy (Riessberger-Galle et al., 2001), která se mění v závislosti na larválním vývoji (Wedenig et al., 2003). Důležitá je zejména vysoká diverzita pylu. Dále bylo zjištěno, že peptidová část mateří kašičky inhibuje růst bakterie *P. larvae* (Bíliková et al., 2001).

Další z alternativních metod kontroly MVP je biologická ochrana založená na přirozené inhibici antagonistickými bakteriemi. V tomto směru se však zatím nedosáhlo uspokojivých výsledků, neboť *P. larvae* produkuje silná antibiotika, která významně omezují přežívání dalších druhů bakterií (Evans and Armstrong, 2005). Vhodnější se zdá být využití bakteriofágů, což jsou viry napadající bakterie. Již bylo vybráno několik bakteriofágů s příznivým účinkem. Díky jejich úzké specializaci neškodí včelám a v případě absence hostitelské bakterie ze včelstva samovolně vymizí (Tsourkas, 2020).

Velký potenciál mají v tomto směru zejména rostlinné silice, jejichž antimikrobiální účinek byl prokázán v laboratorních podmínkách (Calderone et al., 1994; Damiani et al., 2014). Velkou výhodu rostlinných silic představuje jejich široký rozsah účinku a možnost potlačení několika včelích patogenů najednou, včetně kleštíka včelího (Ebert et al., 2007; Genersch, 2010a).

Zajímavý může být i netradiční přístup inspirovaný přirozeným chováním dvou organismů, které představují významné škůdce ve včelařství, a to bakterie *P. larvae* a zavíječ voskový (*Galleria mellonella*). Zavíječ voskový je výjimečný svojí schopností rozkládat včelí vosk a další, svou strukturou podobné, velmi stabilní látky bohaté na alifatické uhlovodíky, jako jsou například plasty (Billen et al., 2020; Bombelli et al., 2017; Kong et al., 2019). Díky tomu se v přírodě podílí na koloběhu živin, plní hygienickou funkci a omezuje akumulaci a šíření patogenů (Ellis et al., 2013). Oba výše uvedené organismy se tedy mohou v přírodě běžně setkat. Vzhledem k velmi vysoké odolnosti spor *P. larvae* je pravděpodobné, že v přírodě existuje nějaký mechanismus zabraňující jejich akumulaci a přemnožení. Nabízí se tedy otázka, zda velmi specifický trávicí systém zavíječe voskového dokáže narušit i spory původce moru včelího plodu. Tímto tématem se zabývá Mráz et al. (2020).

3 Hypotézy a cíle

3.1 Hodnocení vlivu a kvality opylení na kvantitativní a kvalitativní parametry výnosu zimolezu modrého

Citelný úbytek opylovatelů v poslední době může způsobit značné komplikace pěstitelům. Opylování je proces nezbytný pro tvorbu plodů a u hmyzosnubných rostlin je tato činnost zajišťována zejména hmyzími opylovateli. Proto se dílčí část této práce zabývá také vlivem a kvalitou opylení na kvantitativní a kvalitativní parametry výnosu stále populárnější plodiny zimolezu modrého (*Lonicera caerulea*: Caprifoliaceae). Hlavním cílem bylo zjistit, zda opylování přirozenými opylovateli zvyšuje kvalitu a množství vyprodukovaných plodů v porovnání s ručním opylením nebo variantou bez opylení. Dalším cílem bylo porovnat druhovou rozmanitost a početnost včel (Apoidea) přímo na květech zimolezu a v jeho okolí.

Hypotézy:

- Opylování přirozenými opylovateli zvyšuje kvalitu a množství vyprodukovaných plodů.

3.2 Studium diverzity a početnosti včel v různých režimech hospodaření

Kontaminace pesticidy je v dnešní krajině téměř nevyhnutelná a negativně ovlivňuje diverzitu a početnost včel a snižuje i kvalitu včelích produktů. Obecně mezi lokality s nízkým, nebo žádným obsahem reziduí pesticidů patří plochy obhospodařované v ekologickém režimu hospodaření. Proto je snaha množství těchto ploch navyšovat. I přesto je však procentuální zastoupení těchto ploch v krajině velmi nízké. Cílem bylo porovnat diverzitu a početnost včel a dále kontaminační zátěž prostředí rezidui pesticidů mezi lokalitami s ekologickým a konvenčním režimem hospodaření.

Hypotézy:

- Na lokalitě s ekologickým režimem hospodaření se vyskytuje více druhů včel s vyšší početností v porovnání s lokalitou s konvenčním hospodařením.

- Na lokalitě s ekologickým režimem hospodaření se vyskytují rezidua pesticidů v nižší míře než na lokalitě s konvenčním režimem hospodaření.

3.3 Studium vlivu polyfenolů na schopnost detoxikace včel intoxikovaných pesticidem

Často za špatný zdravotní stav včelstev a jejich zimní úhyny může i nedostatečná strava s nízkou pylovou diverzitou často vedoucí až k monodietě s nízkým obsahem fenolických látek. To je zapříčiněno zejména změnou krajinné struktury a managementem hospodaření. V české krajině převažují velké plochy monokultur s minimem meziplodin, mezí, remízků, nebo i plevelů, které představují důležitý zdroj pylu. Cílem tedy bylo zjistit vliv polyfenolů, které se běžně vyskytují v pylu rostlin, na schopnost detoxikace včel a jejich délku života po intoxikaci pesticidem.

Hypotézy:

- Včely krmené stravou s přídavkem polyfenolů mají vyšší aktivitu detoxikačních enzymů.
- Včely krmené stravou s přídavkem polyfenolů přežívají delší dobu.

3.4 Hodnocení diverzity a početnosti vybraných včelích patogenů v různých typech habitatů

Tato část práce se zabývá prevalencí významných patogenů včely medonosné na území České republiky v závislosti na odlišných typech ekosystémů. Včelí patogeny představují velký problém ve včelařství, zejména v dnešní době, kdy jsou včelstva často stresována kontaminanty životního prostředí i podvýživou, což se negativně projevuje na jejich imunitě. Cílem bylo stanovit nejčastěji se vyskytující patogeny včel v České republice a zjistit jejich případný vliv na zimní ztráty včelstev. Dalším cílem bylo ověřit, zda mají ekosystémy s různým stupněm antropogenní zátěže vliv na výskyt a prevalenci těchto patogenů.

Hypotézy:

- Z testovaných patogenů se v České republice vyskytuje nejčastěji *Nosema ceranae*.
- *Nosema apis* se v České republice vyskytuje velmi málo nebo vůbec.

- Typ ekosystému má vliv na výskyt včelích patogenů.
- Patogeny včel ovlivňují přezimování včelstev.

3.5 Testování nových a alternativních možností kontroly vybraných včelích patogenů pomocí rostlinných silic

Nedílnou součástí studie jsou nové a alternativní možnosti kontroly patogenů, které musí být šetrné jak ke včelám, tak i k člověku, nezanechávat rezidua ve včelích produktech a v neposlední řadě vykazovat vysokou účinnost na cílové organismy. Tyto parametry by mohly splňovat určité rostlinné silice, jejichž potenciální využití ve včelařství, zejména ke kontrole parazitického roztoče *Varroa destructor* a entomopatogenní houby *Ascosphaera apis*, je hlavní náplní této práce. Velký důraz je kladen na houbového patogena *A. apis*, u kterého byly nejdříve zkoumány vhodné podmínky pro růst a vývoj a na základě toho proběhla optimalizace kultivace v laboratorních podmínkách. Cílem bylo ověřit často používaná kultivační media a zjistit jejich vliv na růst a reprodukční parametry tohoto patogena. Následně byl testován fungicidní účinek několika vybraných rostlinných silic s cílem zjistit, zda tyto silice mají fungicidní efekt. V případě roztoče *V. destructor* byl sledován akaricidní efekt rostlinných silic a také jejich toxicita vůči včele medonosné. Cílem bylo ověřit, zda mají některé z vybraných rostlinných silic větší toxický efekt na roztoče než na včely a zda by bylo možné jejich využití ve včelařské praxi.

Hypotézy:

- Včelí plod má pozitivní vliv na růst a produkci reprodukčních struktur entomopatogenní houby *A. apis*.
- Některé z vybraných rostlinných silic mají fungicidní nebo akaricidní efekt.
- Některé z vybraných rostlinných silic mají větší toxický efekt na roztoče než na včely.
- Využití rostlinných silic ve včelařské praxi je teoreticky možné.

3.6 Studium nových a alternativních možností kontroly vybraných včelích patogenů pomocí trávicích enzymů zavíječe voskového

Zajímavý může být i netradiční přístup inspirovaný přirozeným chováním dvou organismů, které představují významné škůdce ve včelařství, a to bakterie *Paenibacillus. larvae* a zavíječe voskového (*Galleria mellonella*). Zavíječ voskový je

výjimečný svoji schopností rozkládat včelí vosk a další, svou strukturou podobné, velmi stabilní látky bohaté na alifatické uhlovodíky, jako jsou například plasty. Díky tomu se v přírodě podílí na koloběhu živin, plní hygienickou funkci a omezuje akumulaci a šíření patogenů. Oba výše uvedené organismy se tedy mohou v přírodě běžně setkat. Vzhledem k vysoké odolnosti spor *P. larvae* je pravděpodobné, že v přírodě existuje mechanismus zabraňující jejich akumulaci a přemnožení. Cílem tedy bylo ověřit, zda velmi specifický trávicí systém zavíječe voskového dokáže narušit i spory původce moru včelího plodu.

Hypotézy:

- Trávicí systém zavíječe voskového narušuje spory původce moru včelího plodu.

4 Experimentální část a výsledky

4.1 Hodnocení vlivu a kvality opylení na kvantitativní a kvalitativní parametry výnosu zimolezu modrého

Vlivem opylováním přirozenými opylovateli se zabývá práce Determination of main pollinators of haskap (*Lonicera caerulea*: Caprifoliaceae) and the effect of different controlled methods of pollination on fruit set (Hýbl et al., 2022). Upozorňuje na velký význam přirozených opylovatelů v závislosti na kvalitativních i kvantitativních parametrech výnosu. Ukázalo se, že všechny testované variety zimolezu měly lepší výnosové parametry, včetně rovnoměrného dozrávání, počtu semen i jejich hmotnosti za volného opylení přirozenými opylovateli v porovnání s ručním opylením, a to jak samosprašně tak i pylem z jiných rostlin. U varianty bez opylení v izolaci v některých případech dokonce nedošlo ani k formování semen. To naznačuje silný entomofilní charakter zimolezu. Podobný význam má však opylení přirozenými opylovateli i u mnoha dalších ovocných druhů rostlin.

V poslední době stále rychlejší úbytek hmyzích opylovatelů vede ke snaze omezování pesticidů, k ochraně životního prostředí a podpoře přirozených hmyzích ekotypů. Jde zejména o výsevy květnatých pásů, zvyšování heterogenity krajiny nebo změny managementu zemědělského hospodaření, např. tvorba jednosečných květnatých luk nebo využívání meziplodin a úhorů. V některých oblastech je ale situace tak vážná, že se využívá ručního opylení ovocných stromů a velké úsilí je věnováno i konstrukci malých dronů k podpoře opylování. Tyto aktivity jsou však velmi nákladné a efektivita opylení nízká. Z těchto důvodů je ochrana opylovatelů nezbytná a měla by představovat jeden ze základních pilířů moderního zemědělství.

Publikační výstupy:

Hýbl, M., Mráz, P., Vládek, A, Přidal, A., Polák, O., Šipoš, J. 2022. Determination of main pollinators of haskap (*Lonicera caerulea*: Caprifoliaceae) and the effect of different controlled methods of pollination on fruit set. Agriculture, Ecosystems and Environment – under review.

4.2 Studium diverzity a početnosti včel v různých režimech hospodaření

Kontaminační zátěž prostředí a diverzitu včel v závislosti na různé intenzitě zemědělského hospodaření řeší práce Diversity of bees (Apoidea) and their pesticide contamination in two different types of agricultural management (Hybl et al., 2020). Hlavním cílem bylo zjistit, zda se v krajině obhospodařované šetrnějším způsobem, v tomto případě plochy v ekologickém režimu hospodaření, objevuje vyšší diverzita i početnost hmyzích opylovatelů v porovnání s konvenčním způsobem hospodaření, který je v České republice dominantní. Nejen že byla tato hypotéza průkazně potvrzena, ale navíc bylo v konvenčně obhospodařované krajině nalezeno větší množství reziduí pesticidů. Oproti tomu na plochách v ekologickém režimu hospodaření nebyla nalezena žádná rezidua pesticidů. Právě přítomnost reziduí pesticidů ve včelách by mohl být jeden z hlavních faktorů úbytku početnosti i diverzity opylovatelů. Proto i když je zpravidla ekologické zemědělství náročnější v mnoha aspektech a výnosy jsou často nižší než u konvenčního hospodaření, značnou kompenzací je šetrný přístup k životnímu prostředí a jeho ochrana, což ve výsledku pozitivně ovlivňuje prosperitu jak hmyzích opylovatelů, tak i přirozených nepřátel mnoha škůdců zemědělských plodin. Z tohoto úhlu pohledu je používání značných množství agrochemikálií v konvenčním zemědělství dlouhodobě neudržitelné a je mnohem více žádoucí podporovat jiné systémy hospodaření, jako je například ekologické, integrované, či precizní zemědělství, které kladou důraz na udržitelnost a šetrnost k životnímu prostředí.

Publikační výstupy:

Hybl, M., Mraz, P., Sipos, J., 2020. Diversity of bees (Apoidea) and their pesticide contamination in two different types of agricultural management, in: MendelNet. Brno, pp. 216–221.

4.3 Studium vlivu polyfenolů na schopnost detoxikace včel intoxikovaných pesticidem

Rezidua pesticidů jsou v dnešní době hojně diskutované téma. Jejich odbourávání ze životního prostředí je často velmi problematické a tak se s nimi včely setkávají velmi často. Tato xenobiotika i ve velmi nízké koncentraci zatěžují detoxikační systém včel a způsobují jim stres vedoucí mimo jiné ke snižování

imunity a tím i zvýšení náchylnosti k původcům různých onemocnění. Podporou detoxikace včel od pesticidů se zabývá studie Polyphenols as Food Supplement Improved Food Consumption and Longevity of Honey Bees (*Apis mellifera*) Intoxicated by Pesticide Thiacloprid (Hýbl et al., 2021b). Důraz je kladen na plnohodnotnou výživu včel. K tomu je nezbytná pestrá pylová snůška, která pokryje potřebu všech esenciálních aminokyselin a navíc obsahuje látky fenolické povahy, které jsou nezbytné pro správnou funkci detoxikace. V prováděném experimentu se ukázalo, že tyto látky u včel intoxikovaných pesticidem prodlužují délku života. Navíc působí jako atraktanty, protože zvyšovaly příjem krmiva včelami. To může být vysvětleno i zvýšenou potřebou těchto látek pro detoxikaci, případně podporu imunity, nebo další fyziologické pochody pro dokončení plného vývoje včel. Pro účely pokusu byly totiž vybrány velmi mladé včely do 24 hodin stáří, které ještě nemají všechny obranné mechanismy plně funkční. Pro jejich aktivaci je vyžadován pyl jako hlavní bílkovinná strava s vysokým obsahem polyfenolů. Z těchto důvodů je zřejmé, že vysoká diverzita a dostatek pylových zásob jsou pro včely nezbytné. Na druhou stranu však nebyla prokázána zvýšená aktivita detoxikačních enzymů, jelikož výsledky nebyly přesvědčivé. Genové exprese se mohou odehrávat pouze v určitý okamžik, který nemusel být ve stanoveném časovém intervalu zachycen. Pro přesnější výsledky by pravděpodobně bylo vhodné vybrat více měření v kratších časových intervalech.

Publikační výstupy:

Hýbl, M., Mráz, P., Šipoš, J., Hoštičková, I., Bohatá, A., Čurn, V., Kopec, T., 2021. Polyphenols as Food Supplement Improved Food Consumption and Longevity of Honey Bees (*Apis mellifera*) Intoxicated by Pesticide Thiacloprid. Insects 12, 572.

4.4 Hodnocení diverzity a početnosti vybraných včelích patogenů v různých typech habitatů

Vysoká úroveň imunity včel a jejich schopnost odolávat napadení různými patogeny je v současnosti velmi žádaná a mnohé šlechtitelské programy cílí právě na odolnost včelstev. Ta byla dříve upozaďována před medným výnosem, mírností nebo menší rojivostí a dalšími vlastnostmi, které ve výsledku mohou právě odolnost včelstev snižovat. Tato situace vedla k častějšímu výskytu a prevalenci včelích

patogenů podílejících se na plošných kolapsech včelstev. Výskytem a prevalencí včelích patogenů na území České republiky s ohledem na různé typy ekosystémů a jejich vlivem na přezimování včelstev se zabývá studie Screening of honey bee pathogens in the Czech Republic and their prevalence in various habitats (Mráz et al., 2021a). Ve studii byla sledována přítomnost velkého počtu běžně se vyskytujících včelích patogenů a parazitů, s výjimkou roztoče *Varroa destructor*, který je téměř všudypřítomný. Výsledky naznačují několik zajímavých trendů. Jedním z nich je dominantní výskyt patogenů z čeledi Trypanosomatidae, konkrétně *Lotmaria passim* a *Crithidia mellificae*. Tito parazité byli v minulosti opomíjeni, protože jim nebyl přisuzován negativní vliv na včelstva. Nyní se však ukazuje, že mohou působit negativně a zejména v ko-infekci s parazitickou mikrosporidií *Nosema ceranae* působí velké škody. Právě *Nosema ceranae* je druhý nejčastěji se vyskytující patogen na území České republiky, který díky svojí vyšší virulenci vytlačuje, nebo již kompletně vytlačil původní druh *Nosema apis*, jehož přítomnost nebyla nalezena v žádném z 250 testovaných včelstev. I přesto, že v přiložené studii nebyl průkazně potvrzen vliv ko-infekce *N. ceranae* a parazitů z čeledi Trypanosomatidae na zimní úhyny včelstev, jejich alarmující vysoký výskyt představuje značné riziko a vyžaduje zvýšenou pozornost jak odborných pracovníků, tak i včelařů. Dále bylo zjištěno, že nové formy viru deformovaných křídel, konkrétně DWV-B a DWV-C se vyskytují i v České republice a mají prokazatelně negativní vliv na zimní úhyny včelstev. Prevalence virů je obecně v podmínkách České republiky velmi vysoká. Alespoň jeden virus byl detekován v 74% testovaných včelstev, ve většině případů však bylo nalezeno virů více. Infestace viry je v poslední době další velký problém ve včelařství a to zejména kvůli roztoči *V. destructor*, který slouží jako vektor virů a umožňuje tak jejich snadné šíření.

Publikační výstupy:

Mráz, P., Hýbl, M., Kopecký, M., Bohatá, A., Hoštičková, I., Šipoš, J., Vočadlová, K., Čurn, V. 2021. Screening of honey bee pathogens in the Czech Republic and their prevalence in various habitats. Insects 12, 1051.

4.5 Testování nových a alternativních možností kontroly vybraných včelích patogenů pomocí rostlinných silic

V podmínkách České republiky je roztoč *V. destructor* nepůvodní a včela kraňská, primárně chovaná na našem území, vůči němu nemá vyvinuté přirozené obranné mechanismy. Proto se jedná o jednoho z nejvýznamnějších parazitů včelstev a je mu věnována maximální pozornost. Včelstva napadená roztočem *V. destructor* jsou běžně ošetřována syntetickými akaricidy, jako jsou formamidin amitraz, pyrethroidy tau-fluvalinát a flumethrin, nebo organofosfát kumafos. Tyto látky však zanechávají rezidua ve včelích produktech, působí chronicky na včely i včelí plod a navíc na všechny tyto látky již byla objevena určitá míra rezistence. Z těchto důvodů je snaha o vývoj alternativních metod kontroly roztoče *Varroa*, které musí být účinné, bezpečné pro včely i včelí plod, nezanechávat rezidua, být snadno aplikovatelné a cenově dostupné. Všechny tyto požadavky by mohly splňovat určité rostlinné silice, kterými se zabývá studie Evaluating the Efficacy of 30 Different Essential Oils against *Varroa destructor* and Honey Bee Workers (*Apis mellifera*) (Hýbl et al., 2021a). V první fázi hodnotí akaricidní účinek většího množství rostlinných silic. Ty nejúčinnější silice byly dále testovány společně se včelami v *in vitro* podmínkách a na základě výsledků stanoveny hodnoty LD_{50} a vypočítáno selectivity ratio (poměr mezi LD_{50} na včely a na roztoče). U rostlinných silic s nejvyšší hodnotou selectivity ratio bylo také stanoveno jejich složení a kvantita jejich hlavních složek. Tyto silice, mezi které patří například máta peprná, manuka, dobromysl a vavřín kubébový (litsea) mají velký potenciál ve využití ve včelařské praxi, jelikož mají značný akaricidní účinek a zároveň se jeví být v testovaných koncentracích bezpečné pro včely. V dalších fázích výzkumu však bude nutné ještě vyhodnotit toxicitu rostlinných silic na včelí plod a také stanovit aplikační formy a dávky v poloprovozních podmínkách. Tento trend využívající látky na přírodní bázi a omezení syntetických akaricidů je celosvětový, ale zatím se nepodařilo vyvinout spolehlivý a funkční přípravek. Na Balkáně, zejména v Bosně a Hercegovině je s úspěchem značně používán produkt na bázi rostlinných silic Herba strips, ale jako hlavní účinnou látku obsahuje stále syntetický akaricid flumethrin. Podobný a stále oblíbenější produkt, kompletně bez syntetických akaricidů je Ekopol používaný v Rusku a ve státech východní Evropy, ale jeho účinek je často kontroverzní.

Rostlinné silice mají často i fungicidní efekt, kterého lze využít k potlačování houbového patogena *Ascospahera apis*, případně jako prevence propuknutí onemocnění zvápenatění včelího plodu, které tento patogen způsobuje. Jeho kultivačními podmínkami a virulencí se zabývá studie The effect of artificial media and temperature on the growth and development of the honey bee brood pathogen *Ascosphaera apis* (Mráz et al., 2021b). Výsledky naznačují, že *A. apis* preferuje nižší než pro včelí plod optimální teplotu a proto se častěji vyskytuje ve slabších včelstvech, kde včely nedokáží udržet stálou teplotu. Tento patogen je úzce spjat se včelím plodem a po jeho přidání do kultivačního media, entomopatogenní houba *A. apis* vykazovala výrazně vyšší produkci reprodukčních struktur. Dále se článek zabývá kompeticí různých kmenů mezi sebou a nabízí možné vysvětlení vzniku mumifikovaného plodu bílé barvy, které dosud není zcela objasněno. Všechny tyto poznatky zvyšují porozumění o růstu a vývoji *A. apis* v *in vitro* podmínkách. Na základě těchto výsledků byl testován fungicidní efekt vybraných rostlinných silic. Tímto tématem se zabývají přiložené publikace Inhibitory effect of selected botanical compounds on the honey bee fungal pathogen *Ascosphaera apis* (Mraz et al., 2019) a Antifungal activity of selected botanical compounds on *Ascosphaera apis* (Mráz et al., 2022). Největší fungicidní aktivitu vykazovaly silice z tymiánu, cedrového dřeva, hřebíčku a skořice. Také bylo stanoveno jejich složení, což pomohlo zjistit jednotlivé účinné látky. Na základě tohoto výsledku vznikl předpoklad, že jednotlivé složky ze silic hřebíčku a skořice působí synergicky, jelikož tyto silice vykazovaly větší fungicidní aktivitu než jejich hlavní účinné látky. Výsledky z těchto publikací zabývajících se účinkem rostlinných silic jak na *A. apis*, tak i na *V. destructor* naznačují, že vhodná kombinace olejů by mohla mít široký rozsah působení, čímž by bylo možné redukovat více patogenů/parazitů najednou.

Publikační výstupy:

Mraz, P., Bohata, A., Hostickova, I., Kopecky, M., Zabka, M., Hybl, M., Curn, V., 2019. Inhibitory effect of selected botanical compounds on the honey bee fungal pathogen *Ascosphaera apis*. Proceedings of the MendelNet, Brno, Czech Republic 6–7.

Mráz, P., Hýbl, M., Kopecký, M., Bohatá, A., Konopická, J., Hoštičková, I., Konvalina, P., Šipoš, J., Rost, M., Čurn, V., 2021. The Effect of Artificial Media and

Temperature on the Growth and Development of the Honey Bee Brood Pathogen *Ascosphaera apis*. Biology 10, 431.

Mráz, P., Žabka, M., Hýbl, M., Kopecký, M., Bohatá, A., Tomčala, A., Čurn, V. 2022. Antifungal activity of selected botanical compounds on *Ascosphaera apis*. Industrial Crops and Products – under review.

Hýbl, M., Bohatá, A., Rádsetoulalová, I., Jelínková, I., Vaníčková, A., Mráz, P. 2021. Evaluating the efficacy of 30 different essential oils against *Varroa destructor* and honey bee workers (*Apis mellifera*). Insects 12, 1045.

4.6 Studium nových a alternativních možností kontroly vybraných včelích patogenů pomocí trávicích enzymů zavíječe voskového

Odlišný přístup byl zvolen ke kontrole původce onemocnění moru včelího plodu, kterým se zabývá studie Effect of the digestive proces of the Greater wax moth (*Galleria mellonella*) on the causative agents of American Foulbrood (*Paenibacullus larvae*) (Mraz et al., 2020). Konkrétně se jedná o využití trávicích enzymů motýla zavíječe voskového za účelem poškození velmi odolných spor bakterie *P. larvae*. Tento motýl je ve včelařství považován za škůdce, jelikož se živí na starších včelích plástech tzv. košilkami (zbytky organické hmoty ze svlékání larev), voskem a zásobami pylu. Výjimečná je zejména jeho trávicí soustava, které dokáže rozložit i velmi stabilní látky, jako je např. včelí vosk. Otázkou tedy bylo, zda si poradí i se sporami bakterie *P. larvae*. V experimentu se larvy zavíječe živily na voskových mezistěnách kontaminovaných touto bakterií. Následně byly larvy usmrceny a jejich trávicí trakt rozdělen na 3 části (přední, střední a zadní část). Metodou PCR byla potvrzena přítomnost patogena a kultivačně pak byl stanoven počet kolonií v jednotlivých částech. Zajímavé zjištění bylo, že v zadní části se nevyskytovaly žádné spory, zatímco v přední a střední ano. Nabízí se dvě možná vysvětlení. Vzhledem k mnoha záhybům v trávicí soustavě a pomalé průchodnosti je možné, že se velmi malé spory ještě nestihly dostat do zadních částí traktu. Druhým vysvětlením může být značný sporicidní efekt trávicích enzymů, které nejen že zabraňují sporám vyklíčit (negativní kultivační test), ale dokáží poškodit i jejich genetickou informaci (negativní PCR test). Pro objasnění jsou však nutné další experimenty. Trávicí enzymy zavíječe voskového jsou zajímavé i z pohledu možného využití při biologické degradaci plastické odpadní hmoty, která je za

běžných podmínek velmi pomalá a představuje závažný problém v odpadovém hospodářství i ochraně krajiny.

Publikační výstupy:

Mraz, P., Hybl, M., Kopecky, M., Sipos, J., Ryba, S., Curn, V., 2020. Effect of the digestive process of the Greater wax moth (*Galleria mellonella*) on the causative agents of American Foulbrood (*Paenibacillus larvae*), in: MendelNet. Brno, pp. 413–418.

5 Přílohy

Hodnocení vlivu a kvality opylení na kvantitativní a kvalitativní parametry výnosu zimolezu modrého zahrnuje výsledky publikace ve formě rukopisu v recenzním řízení s názvem:

- Determination of main pollinators of haskap (*Lonicera caerulea*: Caprifoliaceae) and the effect of different controlled methods of pollination on fruit set.

Studium diverzity a početnosti včel v různých režimech hospodaření zahrnuje výsledky recenzované publikace s názvem:

- Diversity of bees (Apoidea) and their pesticide contamination in two different types of agricultural management.

Studium vlivu polyfenolů na schopnost detoxikace včel intoxikovaných pesticidem zahrnuje výsledky publikované v impaktovaném časopise (Q1, IF = 2,8). Název publikace:

- Polyphenols as Food Supplement Improved Food Consumption and Longevity of Honey Bees (*Apis mellifera*) Intoxicated by Pesticide Thiacloprid.

Hodnocení diverzity a početnosti vybraných včelích patogenů v různých typech habitatů zahrnuje publikované v impaktovaném časopise (Q1, IF = 2,8). Název publikace:

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

Testování nových a alternativních možností kontroly vybraných včelích patogenů pomocí rostlinných silic zahrnuje 1 recenzovanou publikaci (uvedená v databázi WoS), 2 publikace v impaktovaném časopise (Q1, IF = 5,1; Q1, IF = 2,8) a 1 rukopis v recenzním řízení. Názvy prací jsou:

- Inhibitory effect of selected botanical compounds on the honey bee fungal pathogen *Ascosphaera apis*
- The effect of artificial media and temperature on the growth and development of the honey bee brood pathogen Ascosphaera apis
- Evaluating the efficacy of 30 different essential oils against *Varroa destructor* and honey bee workers (*Apis mellifera*)
- Antifungal activity of selected botanical compounds on *Ascosphaera apis*

Studium nových a alternativních možností kontroly vybraných včelích patogenů pomocí trávicích enzymů zavíječe voskového zahrnuje výsledky 1 recenzované publikace s názvem:

- Effect of the digestive proces of the Greater wax moth (*Galleria mellonella*) on the causative agents of American Foulbrood (*Paenibacullus larvae*)

5.1

Determination of main pollinators of haskap (*Lonicera caerulea*: Caprifoliaceae) and the effect of different controlled methods of pollination on fruit set

Marian Hybl[a], Petr Mraz[b], Ales Vladek[a], Antonin Pridal[a], Ondrej Polak[c], Jan Sipos[a,*]

[a] Department of Zoology, Fisheries, Hydrobiology and Apiculture, Faculty of AgriSciences, Mendel University in Brno, Zemedelska 1, 613 00 Brno, Czech Republic

[b] Department of Genetics and Agricultural Biotechnology, Faculty of Agriculture, University of South Bohemia in Ceske Budejovice, Studentska 1668, 370 05 Ceske Budejovice, Czech Republic

[c] Department of Crop Science, Breeding and Plant Medicine, Faculty of AgriSciences, Mendel University in Brno, Zemedelska 1, 613 00 Brno, Czech Republic

* Corresponding authors at: Mendel University in Brno, Zemedelska 1, 613 00 Brno, Czech Republic, email: jan.sipos@mendelu.cz

Abstract

The fruit set of haskap is dependent on optimal pollination. Thus, the focus of this study was the effect of pollination in haskap (varieties Viola, Gerda and Sinnaja ptica). The study aimed to verify the impact of different pollination methods on fruit harvest as well as identify the main bee pollinators and compare their significance. The percent fruit set, fruit weight, number of seeds per fruit and percent fruit set in time order were compared for four treatments: free pollination, self-pollination, cross-pollination and no manipulation. In general, the observed production parameters were better under the free pollination treatment. As the cross-pollination method was unable to maximise the fruit set and improve other production parameters, it is clear haskap has an entomophilous character. This proves that an

obscure effect of pollinators on the effectivity of pollination in haskap exists. The results regarding haskap bee pollinators suggest a preference by long-tongued bees belonging to Bombus spp. and *A. mellifera*, despite the fact that short-tongued species of solitary bees were dominant in the haskaps' vicinity. Based on our results, Bombus spp. is the most suitable pollinator in Central Europe conditions. Other experiments have to be conducted to further clarify the reasons for low haskap productivity under isolation with cross-pollination.

Key words: honeysuckle, hand pollination, fruit set, pollinator, *A. mellifera*, Bombus spp.

1. Introduction

The growing human population is increasing demands on agricultural production (Foley et al., 2011). There is concern regarding meeting the growing demand for food while protecting ecosystems and biodiversity (Brussaard et al., 2010; Perfecto and Vandermeer, 2010). Thus, yields will have to increase in the future through either intensifying agriculture or expanding agricultural land or by using a combination of both (Phalan et al., 2011). Given the negative impact that agriculture has on biodiversity (Stoate et al., 2001), steps to increase production need to be in line with sustainable development and the environment (Beddington, 2010; Moudrý et al., 2018). One of the ways to increase crop production is to provide cultivated crops with optimal pollination and thus maximise the quality and quantity of a yield (Garratt et al., 2014).

Although the rate of increase in a yield is highly questionable in some cases due to the great variability of varieties (Garratt et al., 2014; Stanley et al., 2013), even for crops and varieties where a small or slight increase in yield is observed, the nutritional and seed quality is improved significantly (Björkman, 1995). This can be of great economic importance to farmers (Klatt et al., 2014). The optimal level of pollination can directly increase yields, as in

the case of *Vicia faba* (Köpke and Nemecek, 2010), or the content of nutritionally and economically important substances in the fruit, such as in the case of increasing the amount of oil in the fruits of *Brassica napus* (Bommarco et al., 2012). Another factor that is often directly affected by pollination is the shape of the fruit, as is the case with strawberries (Żebrowska, 1998).

Lonicera caerulea var. *kamtschatica* is a slightly tall fruit shrub characterised by two-flowered inflorescences (Bozek, 2012). The flowers are tubular up to 2.5 cm long with fused ovary. The whole fruit is formed by the fusion of two berries (Frier et al., 2016a), forming a co-formation that looks like one berry (Bozek, 2012). Another specific feature of honeysuckle is tolerance to very low temperatures. The plant can withstand up to −40 °C, while flowers can endure up to −8 °C (Řezníček and Salaš, 2004). That allows for very early flowering, which usually lasts two to three weeks (Frier et al., 2016b). The ripening of the fruit also occurs very early, in the temperate zone from the end of May to the beginning of June. The shape of the fruit and the time of its harvest depend on the characteristics of the cultivar (Bozek, 2012).

Haskap originated in the Northern Hemisphere, namely in Asia, Europe and America (Frier et al., 2016a). However, it is spreading outside of this area as a result of its popularity, which is mainly due to the tasty blueberry-like fruits and potential health benefits it offers (Svarcova et al., 2007). Honeysuckle fruits are a valuable source of important nutrients (e.g. anthocyanins, polyphenolic substances, vitamins and minerals) (Gazdik et al., 2008). Their consumption can help to prevent many chronic diseases, including cancer, cardiovascular disease and diabetes mellitus (Svarcova et al., 2007).

Honeysuckle is an attractive source of nectar and pollen for bees (Bozek, 2007; Bożek and Wieniarska, 2006), which suggests its entomophilic demands. This is confirmed by the specific composition of the fruit (double berry), the formation of which needs effective

pollination of both flowers in the inflorescence, requiring specific pollinator behaviour (Bozek, 2012; Frier et al., 2016a; Woodcock et al., 2013). This is typical for species characterised by self-incompatibility and cross-pollination.

The self-incompatibility of honeysuckle has been confirmed by pollination experiments under open and isolated conditions (Bozek, 2012), as well as cyto-embryologically (Boyarskikh, 2017). For this reason, it is assumed that haskap requires cross-pollination of two compatible varieties (Bors et al., 2012).

However, for a comprehensive experimental investigation of pollination requirements, positive (pollinated) controls under isolation must be included in the experiment (Corbet et al., 1991). In addition to the influence of insect pollinators, this will also reveal the effects of foreign pollination and self-pollination, which have yet to be determined.

Solitary bees, Bombus spp. and honey bees are considered suitable pollinators for honeysuckle (Bozek, 2007; Bożek and Wieniarska, 2006), with different pollinators having different efficiencies in pollinating its flowers (Frier et al., 2016a). In the early spring, when honeysuckle blooms, many pollinators are not yet active or able to pollinate at such low temperatures (Frier et al., 2016a). In addition, changes in the composition of bee taxocenoses occur due to different climates (Ottosen, 1987). Given that the relationships between pollinators in connection with the pollination of honeysuckle have not been fully investigated so far (Frier et al., 2016a), it is possible that some synergistic effect in pollination can exist among different taxons of bees (Brittain et al., 2013). Thus, it is appropriate to identify pollinators that are essential for pollination (Leung and Forrest, 2019).

The main objectives of this research are to (i) compare fruit yields (i.e. the number of fruit, the average weight of fruit, the number of seeds per one fruit and ripening/harvesting process) across different pollination methods (i.e. free pollination, self-pollination, cross-

pollination and no manipulation), (ii) compare the composition of bee taxocenosis in the haskaps' vicinity with bee taxocenosis on haskap blossoms during the flowering period, (iii) determine the richness and abundance of bee pollinators (Hymenoptera: Apiformes) and (iv) assess their effectiveness and significance in pollination.

2. Material and methods

2.1. Locations

The experiment was conducted in the spring of 2018 in Žabčice (South Moravian Region, Czech Republic) on the experimental farm of Mendel University where black soils are located. The locality is situated on a flat surface and has, on average, an altitude of 185 m and precipitation of 380–550 mm. The average annual temperature is over 10 °C. In addition to Žabčice, bee collections were also conducted in the foothills of the Bohemian-Moravian Highlands in Příbram in Moravia (South Moravian Region, Czech Republic) on the private haskap orchard. Hilly surfaces with an altitude of 440 m, precipitation of 516–612 mm and annual temperatures over 7.5 °C on average dominate this area. The last observed locality was Lednice (South Moravian Region, Czech Republic) on the private haskap orchard. Flat surfaces with an altitude of 173 m, precipitation of 328–519 mm and annual temperatures over 10 °C on average dominate this area.

2.2 Pollination

2.2.1 Experiment design

Three varieties (Viola, Gerda and Sinnaja ptica) were chosen for this experiment. A soft paintbrush was used for hand pollination. Monitored branches were isolated by a textile net (organdy) to prevent insect pollination. The whole shrubs were covered by a plastic net before the fruits ripened. The observed flowers were indicated by a rainproof marker. With

respect to their compatibility, Tomichka and cultivar Průhonický semenáč (wild seedling) were used for the pollination of the tested varieties (Boyarskikh, 2017).

The experiment was performed in accordance with the principles of Corbet et al. (1991). The following treatments were used: a) free pollination (unlimited access to pollinators), b) self-pollination (hand-pollination by pollen from own shrub, under isolation), c) cross-pollination (hand-pollination by pollen from compatible pollinisers, under isolation) and d) no manipulation (without access to pollinators or manipulation, under isolation).

Each treatment (branches) and each variety (shrubs) consisted of three replications (n=3). One hundred flowers on each branch were indicated by a waterproof marker and included in the experiment. The blooming dates were as follows: 9. 4., 11. 4., 13. 4., 15. 4., 17. 4., 19. 4. and 22. 4. (2018). The fruits was harvested on the following dates: 8. 5., 11. 5., 14. 5., 16. 5., 18. 5. and 21. 5. (2018).

2.2.2 Evaluation of fruit yield

The percent fruit set was evaluated as a proportion of the number of created fruits and monitored inflorescences. For every harvest, the number of fruits, average fruit weight, fruit dimensions (width and length) and average number of seeds were assessed.

2.3 Pollinators

2.3.1 Determination of bees present in the haskaps' vicinity

In order to explore the spectrum of bee species in the areas (Žabčice, Lednice and Příbram na Moravě), Moericke traps were installed in and around the haskap culture. For sampling, bees were collected by an entomoligic hand net in accordance with the Methods of Insect Collection and Preparation (Upton et al., 2010).

2.4.2 Determination of bees at the haskap blossoms

145 Species that pollinated the haskap were identified using by method of transect
146 sampling. Bee visits to haskap blossoms were assessed visually in three transects throughout
147 each field.

148 Bee visits to haskap flowers were recorded. The visits suggesting pollination were
149 visually observed in three repetitions on each locality once a week for three consecutive
150 weeks, which comprised the majority of the blooming period (Petersen et al., 2013). The
151 sampling of bees pollinating haskap was realised between 08:00 and 17:00 (when flowers
152 were open) during appropriate sunny weather. Pollination by bees was observed for a total of
153 10 minutes each by slowly walking around flowering bushes at 10:00, 12:00, 14:00 and 16:00
154 for each locality.

155 Assemblages of bees in the haskaps' vicinity and at the haskap blossoms were
156 established for all sites together. Following this, the lists of species (including bee species and
157 families, their numbers and dominance) were established for both communities. From the
158 data, richness and abundance were established of both communities as well as the Shannon-
159 Weaver index and equitability.

160 The bee assemblages were divided into guilds (*A. mellifera*, bombus spp. and solitary
161 bees) and by tongue length (short-tongued bees and long-tongued bees).

162 The assemblages were compared with each other, and their similarities and attachment
163 were determined depending on the affiliation to the guild or the length of the tongue.

164 The number of flowers visited per minute by bee was determined for A. mellifera and
165 bombus spp. Individual bee was observed when visiting haskap flowers as long as possible.
166 The number of visited flowers were recorded. Assesement of pollinated flowers was
167 determined for all sites.

The flower constancy of *A. mellifera* and Bombus spp. was measured by determining pollen load composition. Several individuals belonging to *A. mellifera* and Bombus spp. were captured directly at the haskap blossoms. Pollen loads were removed from the corbiculae and stored dry in micro-centrifuge vials.

The pollen loads were put into 70% ethanol and shaken and mixed to disperse. The mixture of pollen and ethanol was filtered through filter paper (Whatman 1, in a Buchner funnel, subsequently dried and fixed on a microscope slide using fuchsine gelatine following Dafni et al. [2005]) Pollen grains were recognised and quantified according to Von Der Ohe et al. (2004).

2.3.3 Comparison of number of visited flowers per day between *A. mellifera* and Bombus spp.

To calculate the average number of pollinated flowers (f-t), the following were determined: the attendance rate of the monitored guilds, the average number of visits by one bee per hour (a – n – v), øf/1h = average number of pollinated flowers per hour by one bee, the length of the working day (l – w – d) F.

For both *A. mellifera* and Bombus spp., the number of flowers visited per day was determined for each site separately. Following this, the average was given. The number of flowers visited per day was calculated as (f – t) = (a – n – v) x øf/1m x 60 x (l – w – d).

2.4 Statistical analysis

The comparing of differences in species richness, abundance and assemblage structure between the haskaps' vicinity and the haskap blossoms was based on a repeated sampling design. Therefore, Least-Squares Repeated Measures ANOVA with locality as a random variable was used for this analysis. The Wilcoxon test was used to compare the number of pollinated flowers per minute and the proportion of pollen in the pollen loads among bee

192 guilds (*A. mellifera* and Bombus spp.). The differences in fruit yield parameters between
193 pollination treatments were statistically analysed with a one-way ANOVA, and a post-hoc
194 Tukey test was used to compare differences between pollination treatments. The percent fruit
195 set was transformed by arcsin (x) to improve normality.

196 To compare the assemblage composition between the haskaps' vicinity and the haskap
197 blossoms, partial canonical correspondence analysis (pCCA) with locality as a covariable was
198 used. Bees were sampled sequentially along the growing season. Therefore, we maintained
199 the time autocorrelation of individual records using cyclic shifts. The abundances of each
200 species were transformed by a decimal logarithm. Randomisation was restricted by
201 randomising time records within each locality separately using cyclic shifts. The significance
202 of the canonical axis was tested with a restricted Monte Carlo permutation test for the time
203 series with 2,000 permutations. To describe how the ecological traits were distributed
204 between the haskaps' vicinity and the haskap blossoms, community-weighted means (CWMs)
205 were calculated and passively displayed in an ordination diagram. CWMs were calculated as
206 the mean trait value for each sample weighted by the relative abundance of the species sharing
207 a given trait. The arrow direction in the ordination diagram indicates the occurrence of
208 dominant trait values in the assemblages. All ordination analyses were conducted by the
209 statistical software CANOCO, v. 5.

210 To reveal whether the species composition in the haskaps' vicinity determined the
211 species assemblage at the haskap blossoms, we used co-correspondence analysis. This type of
212 analysis relates two correspondence analyses to maximise covariance among them (ter Braak
213 and Schaffers, 2004). Therefore, we were able to identify and test the patterns that were
214 common to species assemblage in the haskaps' vicinity and at the haskap blossoms and also
215 weather haskap blossom assemblage were determined by bee assemblages in the haskaps'
216 vicinity.

3. Results

3.1 Yield

Differences in the percent fruit set are depicted in Figure 1. The differences were highly significant in the case of all tested varieties: Gerda ($F_{3,8} = 41.99$; $p < 0.001$), Sinnaja ptica ($F_{3,8} = 18.88$; $p < 0.001$) and Viola ($F_{3,8} = 34.53$; $p < 0.001$). The significantly highest production was found under free pollination for all three varieties (Tukey test, $p < 0.05$). Significant change between isolation treatments in fruit production was not observed in the varieties Gerda and Sinnaja ptica. However, significant difference between treatments in isolation was observed in the case of the variety Viola, which reached the significantly highest yield under the cross-pollination treatment.

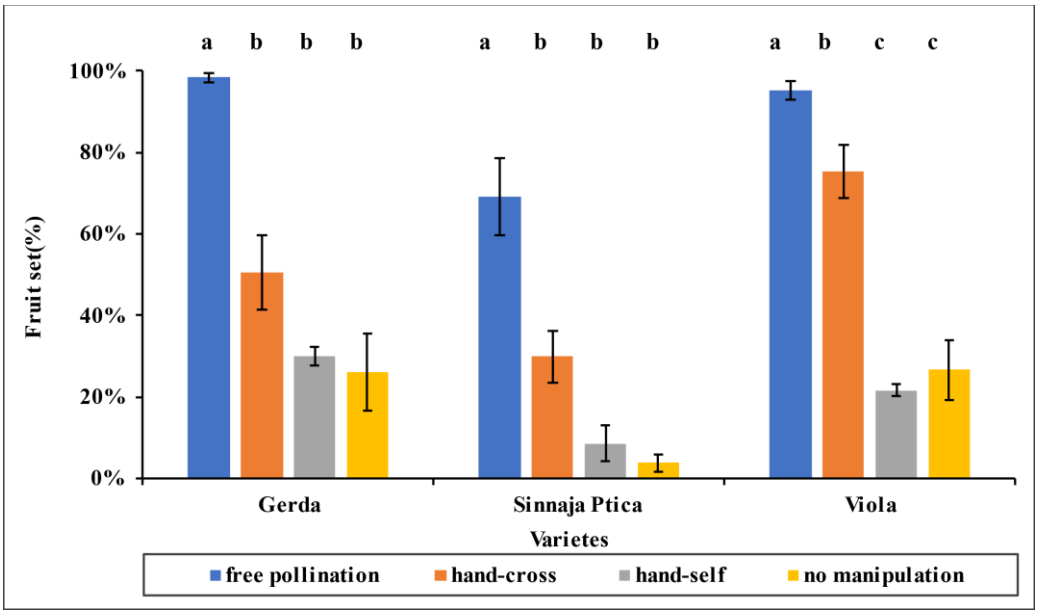


Figure 1: Percent fruit sets according to variety and pollination method. The Tukey test was used to determine differences in the fruit sets between particular pollination methods for each variety. Error bars represent a 95% confidence interval. Significant differences ($p < 0.05$) among treatments are indicated by different letters.

The differences in the fruit weight were highly significant in the varieties Gerda ($F_{3,8} = 22.12$; $p < 0.001$) and Viola ($F_{3,8} = 56.39$; $p < 0.001$), and the significantly heaviest

fruits were recorded under free pollination treatment for both varieties (Tukey test, $p < 0.05$) (Figure 2). The average fruit weight in the case of Viola under isolation was significantly higher only with the cross-pollination treatment. In the case of Gerda, only treatment without manipulation was significantly different from cross-pollination. Regarding Sinnaja ptica ($F_{3,8} = 15.64$; $p < 0.001$), heavier fruit was observed in the case of free pollination compared to other treatments, but only in the cases of no manipulation and self-pollination were the differences significant.

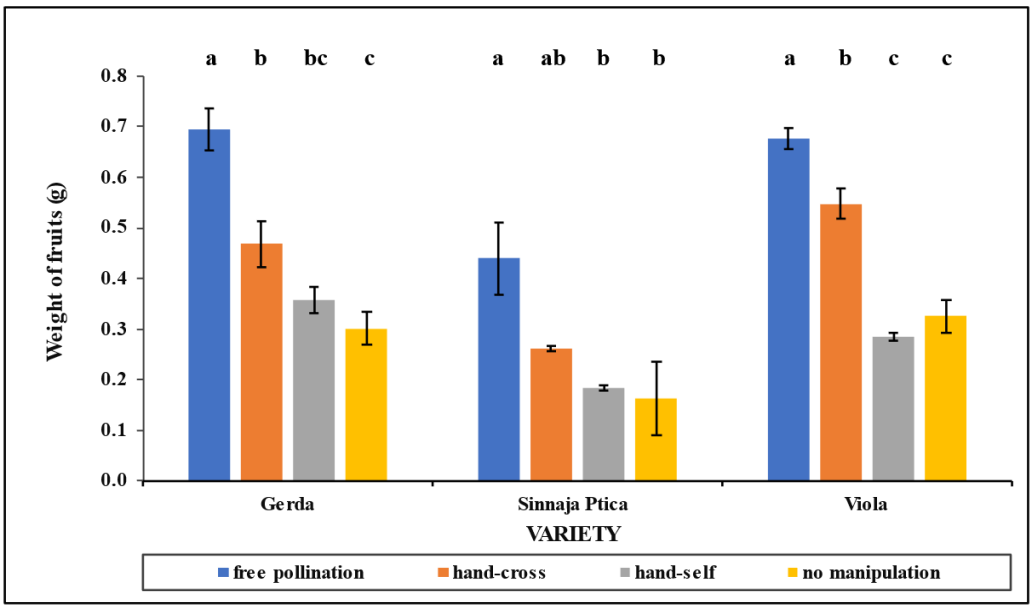


Figure 2: Average weights of fruit according to variety and pollination method. The Tukey test was used to determine differences in fruit weight between particular pollination methods for each variety. Error bars represent a 95% confidence interval. Significant differences ($p < 0.05$) among treatments are indicated by different letters.

In regard to the number of seeds per fruit, significant differences were observed between pollination methods. Figure 3 shows that the number of seeds was several times higher under free pollination than under cross-pollination for all observed varieties. Higher seed counts within isolated treatments have always been observed in cross-pollination, but only in the cases of Gerda and Viola were the differences significant (Tukey test, $p < 0.05$).

Compared to the self-pollination and no manipulation treatments, the average number of seeds in the case of self-pollination was higher for all varieties. In the case of Sinaja ptica, no seed formation was even observed in the no manipulation treatment.

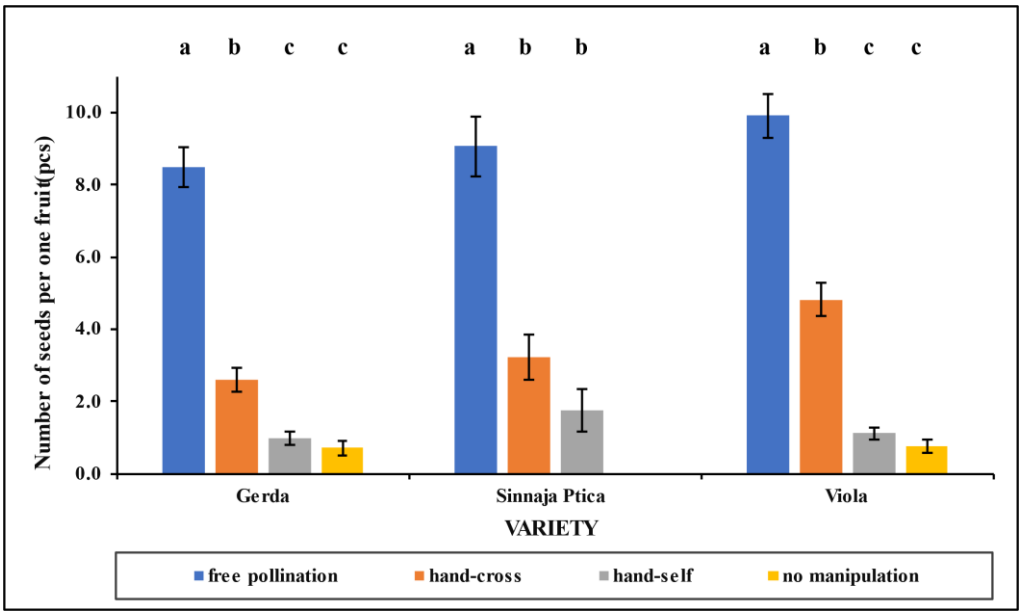


Figure 3: Average numbers of seeds per fruit according to variety and pollination method. The Tukey test was used to determine differences in the number of seeds between particular pollination methods for each variety. Error bars represent a 95% confidence interval. Significant differences ($p < 0.05$) among treatments are indicated by different letters.

The free pollination treatment resulted in earlier harvesting for all varieties (Appendix C). The highest harvesting intensity (i.e. the highest fruit set per harvest day) was achieved under the free pollination treatment. The harvest tops came later (by about one harvest day) under isolation compared to under free pollination and at the same time compared with these three treatments. The duration of harvesting was about two to three days longer under the isolated treatments.

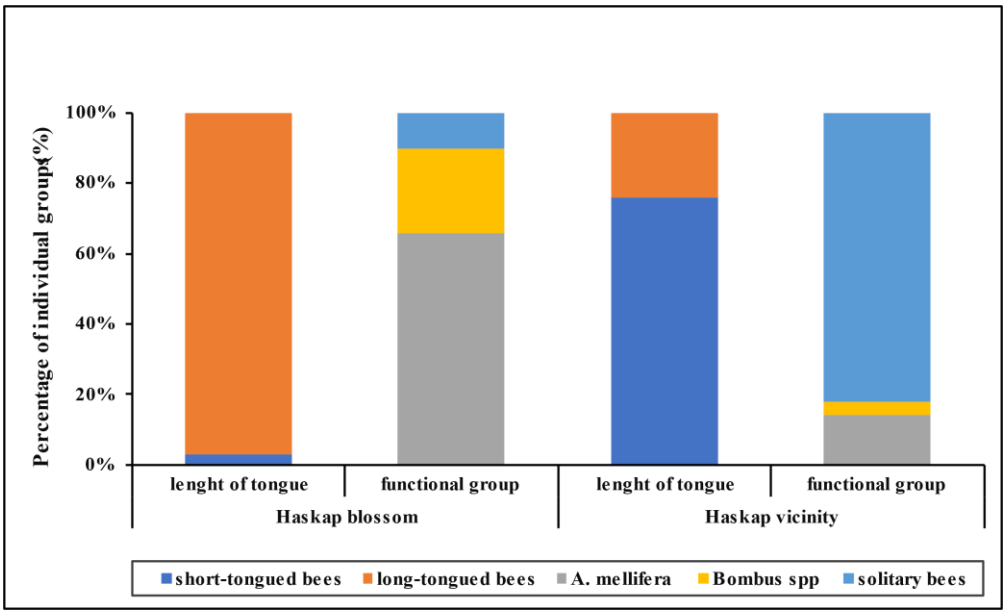
3.2 Pollinators of haskap

3.2.1 Bees in the haskaps' vicinity

In the haskaps' vicinity, a total of 49 species of bees from five families (Apidae, Andrenidae, Colletidae, Halictidae and Megachilidae) were registered (Appendix A, supplementary material). Within the taxonomical groups, the majority of the observed individuals were solitary bees (82.14%), represented by *Andrena flavipes* (eudominant), *Andrena gravida* (dominant) and *Evylaeus malachurus* (dominant). There were fewer individuals observed in the case of *A. mellifera* (14.29%, eudominant), and there were even fewer individuals belonging to Bombus spp. (3.57%; represented by five species, of which *Bobus terrestris* was the only subdominant species). The remaining four species only occurred subrecessively (Appendix A, supplementary material). In addition, significant difference was observed regarding the proportion of long-tongued and short-tongued bees, with most bees belonging to the latter group (75.89%) and less than a quarter belonging to the former group (24.1%; see Figure 4).

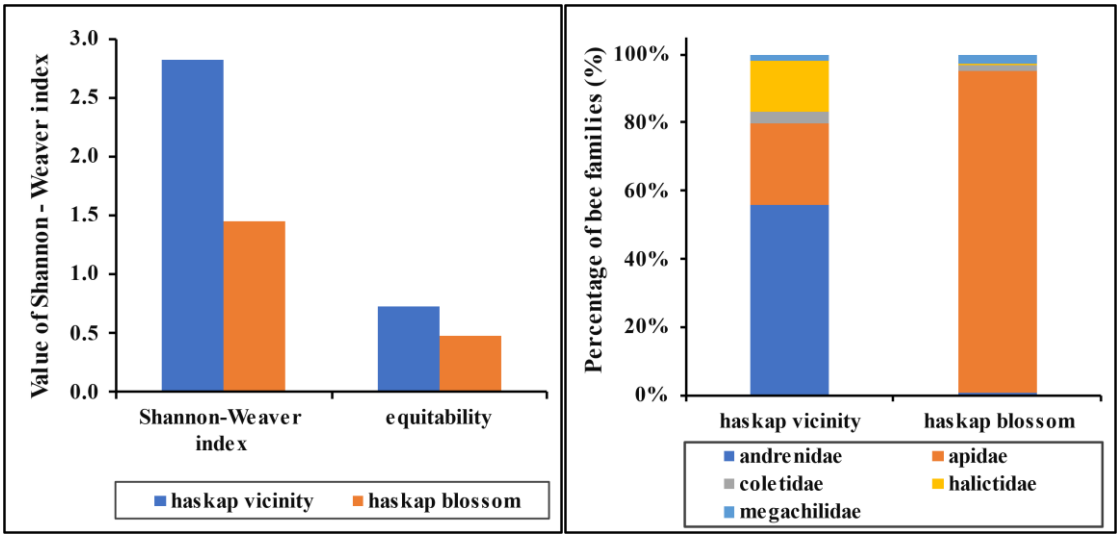
3.2.2 Bees at the haskap blossoms

At the haskap blossoms, 21 species of bees from five families (Apidae, Andrenidae, Colletidae, Halictidae and Megachilidae) were registered (Appendix B, supplementary material). Within the functional groups, the majority of the observed individuals were *A. mellifera* (65.66%, eudominant). A significant proportion of the bees involved in pollination were Bombus spp. (23.99%), represented by nine species, of which the most abundant were *Pyrobombus pratorum* (eudominant) and *Bombus terrestris* (dominant). The smallest proportion of individuals consisted of solitary bees (10.35%), represented by 12 species, of which only the most abundant species, *Anthophora plumipes*, was subdominant. The rest of the solitary bees were even less represented (Appendix B, supplementary material). The largest difference was observed in the proportion of long-tongued and short-tongued bees, with the vast majority of bees falling under the former classification (96.97%) and only a fraction falling under the latter (3.03%; see Figure 4).



292

293 *Figure 4: Barplots showing proportions of pollinator species among taxonomical groups.*



294

295 *Figure 5: Value of Shannon-Weaver index and equitability for species assemblage in the*

296 *haskaps' vicinity and at the haskap blossoms (left). Figure 6: Comparison of the percentages*

297 *of bee families between the haskaps' vicinity and haskap blossoms (right).*

298 **3.2.3 Comparison of bee assemblage in the haskaps' vicinity and at the haskap blossoms**

299 Figure 4 shows the difference in the composition of the pollinator assemblage and

300 species richness in the haskaps' vicinity and at the haskap blossoms (Figure 7). While the bee

301 assemblage in the haskaps' vicinity had 49 species, the composition of pollinators of haskap

blossom consisted of 21 species. We also observed differences in the proportions of bee families, which were more balanced in the haskaps' vicinity compared to the assemblage at the haskap blossoms (Figures 5 and 6). Higher evenness in the abundance distribution across species in the haskaps' vicinity in comparison to at the haskap blossoms was also confirmed by the measurements of the equitability index (Figure 5). Moreover, based on the analysis, species diversity ($F_{1,8}$= 18.06, $p < 0.01$) was significantly higher in the haskaps' vicinity compared to at the haskap blossoms (Figure 7). However, we did not find any significant difference in regard to species abundance (Figure 8).

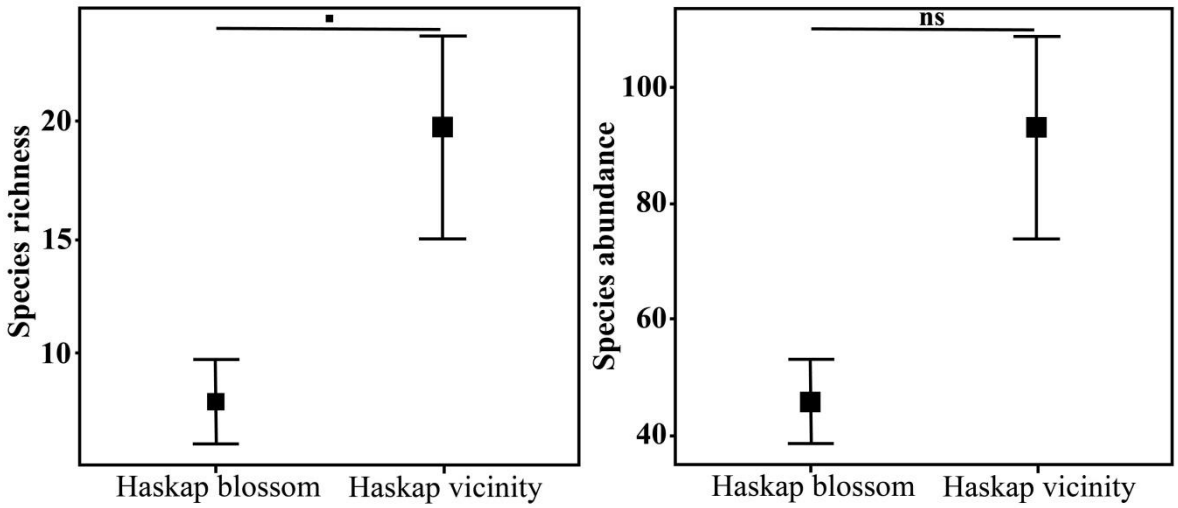


Figure 7: Species richness (left). Figure 8: Comparison of species abundance (right) between different places of catch. Least-Squares Repeated Measures ANOVA was used as a significance test.

Based on the co-correspondence analysis, the species composition at the haskap blossoms was significantly associated with the species in the haskaps' vicinity (Figures 9 and 10) (test on first axis: lambda = 0.256, $p < 0.01$; test on all axes: trace = 0.347, $p = 0.011$). While short-tongued bees (largely represented by solitary bees) were tied to the haskaps' vicinity, long-tongued bees (represented primarily by Bumbus spp. and *A. mellifera*) were tied

to the haskap blossom assemblage (Figure 11). The legend to Figures 9, 10 and 11 is in Appendix D.

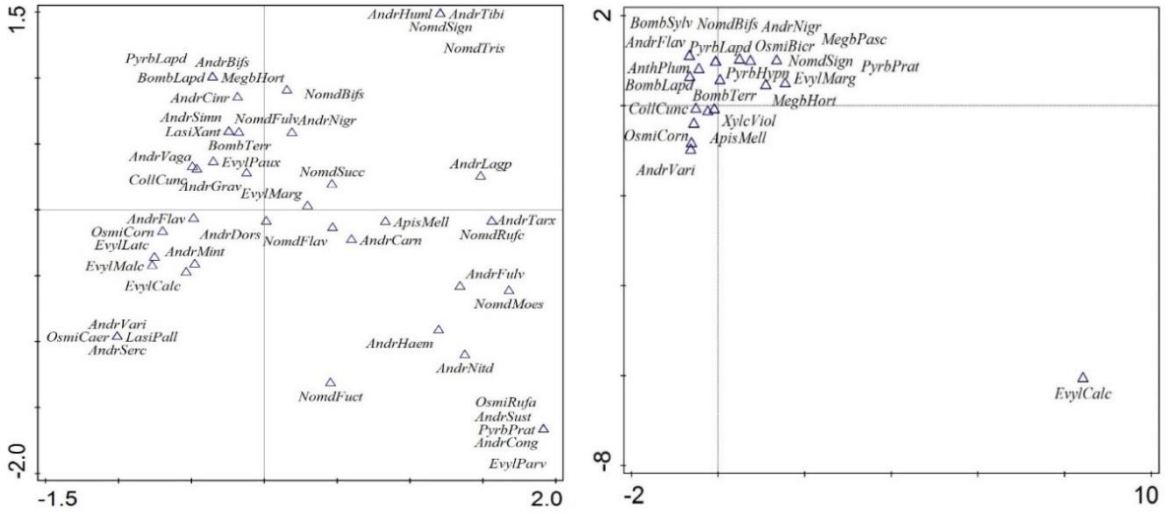


Figure 9: Diagrams of co-correspondence analysis showing species composition in the haskaps' vicinity (left). Figure 10: Diagrams of co-correspondence analysis showing species composition at the haskap blossoms (right).

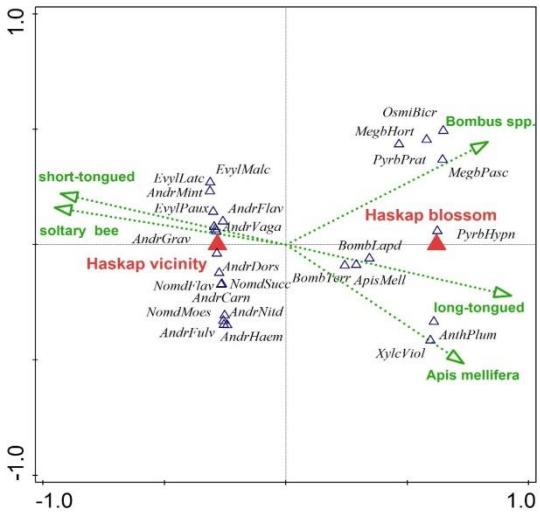


Figure 11: pCCA biplot with the locality as a covariable showing differences within bee assemblages according to the haskaps' vicinity and the haskap blossoms. CWMs of species characteristic were passively projected onto the ordination diagram.

3.2.4 Comparison of pollination effectivity between main haskap pollinators

The speed rate of pollination varied considerably between *A. mellifera* and Bombus spp. While individual *A. mellifera* visited an average of 7.53 flowers per minute, Bombus spp. visited an average of 28.27 flowers per minute. The speed of work therefore significantly differed in both groups ($p < 0.001$) (Figure 12).

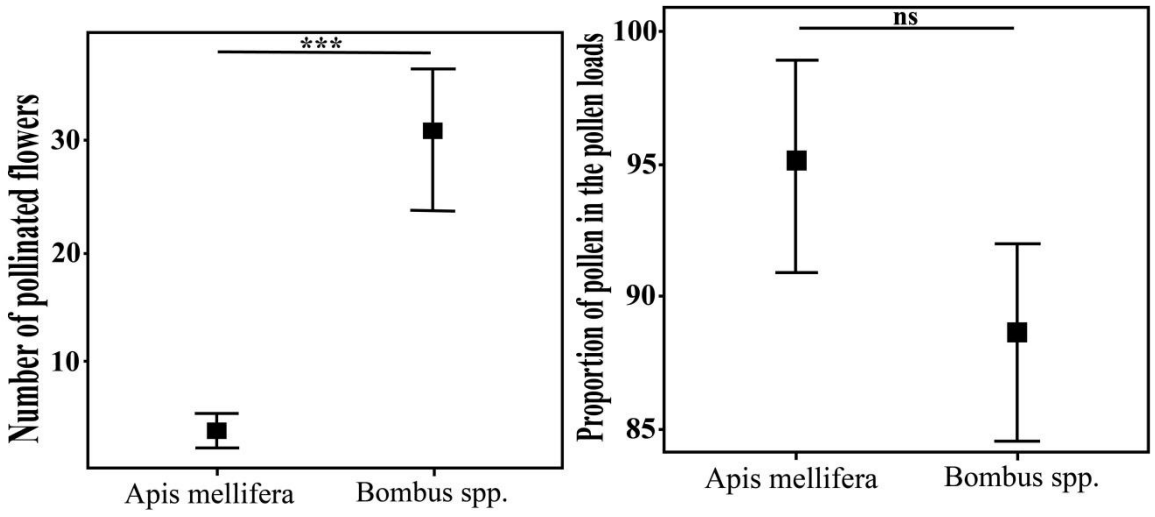


Figure 12: Number of pollinated flowers per minute (left). Figure 13: Proportion of lonicera pollen in the pollen loads (right). The Wilcoxon test was used as a significance test.

Another factor that influenced the efficiency and significance of pollinators was the proportion of pollen transmitted by the pollinator. In the case of *A. mellifera* (95.5%), a significantly higher transmission of haskap pollen grains was not observed in comparison to Bombus spp. (75.5%) ($p = 0.7438$) (Figure 13). Individuals of *A. mellifera* were observed from about 09:00 to 16:00, so the length of their working day was set at an average of seven hours. Bombus spp. were observed from 08:00 to 17:00, so the length of their working day was set at nine hours. From all recorded flower visits at all habitats, the average number of individuals visiting honeysuckle flowers per unit of time was calculated for *A. mellifera* and Bombus spp. The average number of visits was higher in the case of *A. mellifera* compared to Bombus spp. Nevertheless, the average number of flowers visited per hour per bee was many times higher in the case of Bombus spp. Furthermore, the working day length was longer for

Bombus spp. (nine hours) than for *A. mellifera* (seven hours). Considering these results, Bombus spp. could participate in more flower visits even in smaller numbers (Table 1).

Table 1: Comparison of average number of visited flowers per day between A. mellifera and Bombus spp.

A. mellifera	a – n – v	109.5
	øf/1m	7.53
	l – w – d	7
	f – v – d	346,305
Bombus spp.	a – n – v	28
	øf/1m	28.27
	l – w – d	9
	f – v – d	427,442

Legend: a – n – v = average number of visits (one hour), øf/1m = average number of visited flowers per minute per bee, l – w – d = length of working day, f – v – d = number of flowers visited per day.

4. Discussion

The significantly highest percent fruit set was observed in all varieties of haskap under free pollination. In contrast, percent fruit set under isolation conditions without manipulation was three times lower for Viola and Gerda and even lower in the case of Sinnaja ptica. The same trend was detected in regard to the weights of fruit. This is consistent with the results obtained by Bozek (2012). Free pollination had the greatest effect on the number of seeds per fruit. Regarding the varieties Gerda and Viola, the numbers of seeds per fruit were found to be more than ten times higher under free pollination compared to under isolation conditions without manipulation. In the case of Sinaja ptica under isolation and without manipulation,

seed formation did not occur at all. This suggests a higher possible susceptibility of different varieties to seed formation depending on insect pollination. This finding is in line with Bozek (2012) and applies to other fruit species (Garratt et al., 2014). The positive effect of insect pollinators is also undeniable with respect to uneven ripening, which leads to harvest complications in the case of treatments under isolation. This pattern of optimal pollination causing shorter harvest periods is known to also be the case for other crops (Racys and Montviliene, 2005; Williams, 1985).

We observed that cross-pollination did not maximise the fruit set. This indicates that there can be a 'pollinator' factor or factors that increase the efficiency of pollination. This is reflected in all monitored yield parameters (fruit set, weight and number of seeds). There are possible explanations: haskap flowers require a) greater amounts of pollen grains transported on the stigma, which is ensured by a higher number of flower visits (Frier et al., 2016b; Rader et al., 2009) and/or b) higher demands on the specificity of polliniser necessary to achieve compatibility (Bors et al., 2012; Boyarskikh, 2017). These factors seem to be the main cause of the differences in fruit and seed productivity between the cross-pollination and self-pollination treatments. The differences in the fruit set, weight of fruit and number of seeds per fruit were dependent on the variety.

We did not find any significant difference between the self-pollination and without manipulation treatments in regard to the fruit set and weight of fruit. The same pattern was observed concerning the number of seeds per fruit, with the exception of the case of Sinnaja ptica, in which no seeds were created under treatment without manipulation. This suggests a limited level of self-compatibility, which is in line with Bors et al. (2012). Flowers without manipulation were pollinated by an expected internal process of self-transfer of their own pollen grains from anther to stigma (Frier et al., 2016b). This self-fertilisation process likely maximised fruit productivity by itself; therefore, the hand-transferred pollen grains inside of

the same flower by paintbrush were surplus. However, there was a difference in the case of cross-pollination compared with self-pollination in that the transfer of foreign pollen increased fruit productivity, as previously discussed. To explain the low productivity of the haskap in the case of the cross-pollination treatment, it would be appropriate to perform further experiments.

A relatively high number of bee species was observed in the localities in the haskaps' vicinity. Short-tongued species, represented mainly by individuals from the Andrenidae family, comprised the majority of these bees. In contrast, the assemblage of bees observed at the haskap blossoms was dominated by long-tongued bees that belonged mainly to the Apidae family. Short-tongued bee species barely participated in pollination (Figure 7). This is also reflected in the declining values of the Shannon-Weaver index and equitability, which are significantly lower for the bee assemblages observed at the haskap blossoms than for the apidofauna observed in the haskaps' vicinity (Figure 8). Despite the species richness of the bees pollinating haskap being significantly lower compared to the number of species occurring in the haskaps' vicinity, their abundance was not significantly reduced (see Figure 9). These results indicate a high competitive potential of long-tongued bees due to the deep flowering corolla of haskap flowers (Hummer et al., 2012). This is supported by the fact that haskap was originally a circumpolar species, meaning Bombus spp., the classical representatives of long-tongued bees, are by far the best adapted pollinators (Kevan et al., 1993). However, in conditions outside the Arctic and subarctic zones, *A. mellifera* also seem to be another important pollinator of haskap, perhaps even the most abundant pollinator in warmer conditions. This is in line with the findings of Frier et al. (2016a) and Olmstead (2019).

Although the haskap-pollinating bee assemblage differed significantly from the bee assemblage in the haskaps' vicinity, the species composition of the haskap-pollinating

assemblage was logically directly influenced by the bee assemblage in the haskaps' vicinity. Therefore, there were many species observed on haskap flowers that were also present in the bee assemblages observed in the haskaps' vicinity (Figure 10). However, the species common to both assemblages differed significantly in abundance (Figure 7). A different trend was observed in each assemblage: while short-tongued bee species represented by the solitary bee guild were associated with the bee assemblages in the haskaps' vicinity, long-tongued bee species were linked with the bee assemblages at the haskap blossoms, particularly *A. mellifera* and Bombus spp. (Figure 11). A similar trend was observed by Leung and Forrest (2019). The nesting of various native species of solitary bees was observed in the haskap orchard, of which only a few long-tongued species participated in pollination, with the vast majority of the solitary bees preferring other food sources.

We also determined a difference among the main guilds of haskap pollinators (*A. mellifera* and Bombus spp.) in regard to the number of flowers visited by an individual per unit of time, pollen load composition (indicating the flower constancy of the guilds and working day length (which is closely related to the number of potentially visited/pollinated flowers per day). Based on this, we compared the effectiveness and importance of both guilds for haskap pollination. While one *A. mellifera* worker visited an average of 7.5 flowers per minute, the average Bombus spp. was able to visit almost four times more flowers in the same amount of time (Figure 12l). Although *A. mellifera* were expected to have a higher degree of floroconstancy compared to Bombus spp. and thus a significantly higher proportion of transported haskap pollen (i.e. a lower probability of contamination of stigma with pollen from different plant species), this was not confirmed, and a high proportion of haskap pollen was observed in both guilds (Figure 12r). Both guilds seemed to be extremely effective compared to the *Osmia lignaria* (long-tongued solitary bees considered a natural pollinator of haskap) mentioned in the study of Frier et al. (2016a), where the average pollen load was only

5% of haskap pollen. After observing the activity of individuals belonging to the guilds of *A. mellifera* and Bombus spp., the working day was set at two hours longer for Bombus spp. This is due to the ability of bumblebees to function even at significantly lower temperatures than *A. mellifera* can (Frier et al., 2016a; Lundberg, 1980).

The most numerous bee guild that participated in haskap pollination was *A. mellifera*, which is consistent with Frier et al. (2016a) and Olmstead (2019). When calculating the potentially visited/pollinated flowers per day, using the data obtained, however, it turned out that despite the significantly higher involvement of *A. mellifera*, bumblebees were still able to visit more flowers. This result reflects their ability to visit many flowers per unit of time and the longer length of their working day. Frier et al. (2016a) further stated that bumblebees are able to transfer more pollen to the stigma per visit to the haskap flower and thus ensure complete fertilisation in a single visit, unlike *A. mellifera*, which have to visit the flower several times for complete fertilisation. However, the sexual parts of the flowers are active for several days, even in the case of pollination (Frier et al., 2016b), meaning there is a relatively high probability that many flowers will be visited/pollinated more than once.

Some studies have assessed the suitability of pollinators by body size, which has a crucial impact on circadian rhythms (Hoehn et al., 2008). In the present study, however, due to the flower biology (length of the corolla and the long-term activity of the sex parts of the flower), we focused on the suitability of pollinators according to the length of the tongue, following the pattern of Fontaine et al. (2006). While Frier et al. (2016a) assumed that the haskap uses a generalist pollination strategy to maximise the use of the limited number of pollinators available in the spring based on the characteristics of the flower, we revealed that in the relationship between plants and pollinators, haskap primarily employs long-tongued generalist bees.

To ensure optimal pollination, a supply of social bee species such as bumblebees and *A. mellifera*, which are the most effective pollinators of haskap, is recommended. The benefits of these species are their easy displaceability, numerous colonies and affordability. A supply of commercially bred pollinators is particularly recommended when the community of native pollinators is disturbed and there is a risk of reducing the fruit set (Hoehn et al., 2008; Klein et al., 2007). Furthermore, these bees species are relatively more resistant against anthropogenic changes in landscape (Hybl et al., 2020).

5. Conclusion

The harvest parameters of haskap (percent fruit set, fruit weight, number of seeds per fruit and term and duration of harvest) were found to be most optimised under free pollination compared to under other treatments, including cross-pollination. This proves that some obscure effect of pollinators on the effectivity of the pollination process in haskap must exist.

Despite the dominant occurrence of short-tongued bee species belonging to solitary bees, the pollination of haskap was almost exclusively due to long-tongued bee species, mainly represented by Bombus spp. and *A. mellifera*. Thus, it can be assumed that the most important bees for pollination and yield generation are social species of bees that are traded, great in number and easily movable.

The most abundant bee species observed during the pollination of haskap was *A. mellifera*. Although significantly fewer *Bombus* spp. were observed on the flowers, it is assumed that they did more work in total than *A. mellifera*, mainly due to their higher work efficiency and longer working day.

In warmer areas with sufficient density, *A. mellifera* can likely become the most important pollinator of haskap. During a high flight, they can pollinate more flowers per day than Bombus spp. (Frier et al., 2016a). This could be particularly valid in areas with strongly reduced occurrences of wild pollinators (Hoehn et al., 2008).

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

We thank to Libor Dokoupil, Magdalena Tvrznikova (both from Mendel University in Brno) for provided haskap plantation and Vojtech Reznicek (Mendel University in Brno) for pomological consultations.

References

Beddington, J., 2010. Food security: contributions from science to a new and greener revolution. Philosophical Transactions of the Royal Society B: Biological Sciences 365, 61–71.

Björkman, T., 1995. Role of honey bees (Hymenoptera: Apidae) in the pollination of buckwheat in eastern North America. Journal of Economic Entomology 88, 1739–1745.

Bommarco, R., Marini, L., Vaissière, B.E., 2012. Insect pollination enhances seed yield, quality, and market value in oilseed rape. Oecologia 169, 1025–1032.

Bors, B., Thomson, J., Sawchuk, E., Reimer, P., Sawatzky, R., Sander, T., Kaban, T., Gerbrandt, E., Dawson, J., 2012. Haskap Berry Breeding and Production. University of Saskatchewan.

Boyarskikh, I., 2017. Features of Lonicera caerulea L. reproductive biology. Agricultural biology.

Bozek, M., 2012. The Effect of Pollinating Insects on Fruiting of Two Cultivars of Lonicera caerulea L. Journal of Apicultural Science 56, 5.

512 Bozek, M., 2007. Pollen productivity and morphology of pollen grains in two cultivars of
513 honeyberry [Lonicera kamtschatica [Sevast.] Pojark.]. Acta Agrobotanica 60.

514 Bożek, M., Wieniarska, J., 2006. Blooming biology and sugar efficiency of two cultivars of
515 Lonicera kamtschatica (Sevast.) Pojark.

516 Brittain, C., Williams, N., Kremen, C., Klein, A.-M., 2013. Synergistic effects of non-Apis
517 bees and honey bees for pollination services. Proceedings of the Royal Society B: Biological
518 Sciences 280, 20122767.

519 Brussaard, L., Caron, P., Campbell, B., Lipper, L., Mainka, S., Rabbinge, R., Babin, D.,
520 Pulleman, M., 2010. Reconciling biodiversity conservation and food security: scientific
521 challenges for a new agriculture. Current opinion in Environmental sustainability 2, 34–42.

522 Corbet, S.A., Williams, I.H., Osborne, J.L., 1991. Bees and the pollination of crops and wild
523 flowers in the European Community. Bee world 72, 47–59.

524 Dafni, A., Kevan, P.G., Husband, B.C., 2005. Practical pollination biology. Practical
525 pollination biology.

526 Foley, J.A., Ramankutty, N., Brauman, K.A., Cassidy, E.S., Gerber, J.S., Johnston, M.,
527 Mueller, N.D., O'Connell, C., Ray, D.K., West, P.C., 2011. Solutions for a cultivated planet.
528 Nature 478, 337–342.

529 Fontaine, C., Dajoz, I., Meriguet, J., Loreau, M., 2006. Functional diversity of plant–
530 pollinator interaction webs enhances the persistence of plant communities. PLoS biology 4,
531 e1.

532 Frier, S D, Somers, C.M., Sheffield, C.S., 2016. Floral longevity, nectar production, pollen
533 release, and stigma receptivity in Haskap (Lonicera caerulea). Journal of Pollination Ecology
534 19, 81–87.

Frier, S D, Somers, C.M., Sheffield, C.S., 2016. Comparing the performance of native and managed pollinators of Haskap (Lonicera caerulea: Caprifoliaceae), an emerging fruit crop. Agriculture, Ecosystems & Environment 219, 42–48.

Garratt, M.P., Breeze, T.D., Jenner, N., Polce, C., Biesmeijer, J.C., Potts, S.G., 2014. Avoiding a bad apple: Insect pollination enhances fruit quality and economic value. Agriculture, ecosystems & environment 184, 34–40.

Gazdik, Z., Reznicek, V., Adam, V., Zitka, O., Jurikova, T., Krska, B., Matuskovic, J., Plsek, J., Saloun, J., Horna, A., 2008. Use of liquid chromatography with electrochemical detection for the determination of antioxidants in less common fruits. Molecules 13, 2823–2836.

Hoehn, P., Tscharntke, T., Tylianakis, J.M., Steffan-Dewenter, I., 2008. Functional group diversity of bee pollinators increases crop yield. Proceedings of the Royal Society B: Biological Sciences 275, 2283–2291.

Hummer, K.E., Pomper, K.W., Postman, J., Graham, C.J., Stover, E., Mercure, E.W., Aradhya, M., Crisosto, C.H., Ferguson, L., Thompson, M.M., 2012. Emerging fruit crops, in: Fruit Breeding. Springer, pp. 97–147.

Hybl, M., Mraz, P., Sipos, J., 2020. Diversity of bees (Apoidea) and their pesticide contamination in two different types of agricultural management, in: MendelNet. Brno, pp. 216–221.

Kevan, P., Tikhmenev, E., Usui, M., 1993. Insects and plants in the pollination ecology of the boreal zone. Ecological Research 8, 247–267.

Klatt, B.K., Holzschuh, A., Westphal, C., Clough, Y., Smit, I., Pawelzik, E., Tscharntke, T., 2014. Bee pollination improves crop quality, shelf life and commercial value. Proceedings of the Royal Society B: Biological Sciences 281, 20132440.

558 Klein, A.-M., Vaissiere, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen,
559 C., Tscharntke, T., 2007. Importance of pollinators in changing landscapes for world crops.
560 Proceedings of the royal society B: biological sciences 274, 303–313.

561 Köpke, U., Nemecek, T., 2010. Ecological services of faba bean. Field crops research 115,
562 217–233.

563 Leung, M.C.-Y., Forrest, J.R., 2019. Insect pollinators of haskap (Lonicera caerulea L.:
564 Caprifoliaceae) in subarctic Canada. Open Agriculture 4, 676–683.

565 Lundberg, H., 1980. Effects of weather on foraging-flights of bumblebees (Hymenoptera,
566 Apidae) in a subalpine/alpine area. Ecography 3, 104–110.

567 Moudrý, J., Bernas, J., Konvalina, P., Ujj, A., Manolov, I., Stoeva, A., Rembiałkowska, E.,
568 Stalenga, J., Toncea, I., Fitiu, A., 2018. Agroecology Development in Eastern Europe—Cases
569 in Czech Republic, Bulgaria, Hungary, Poland, Romania, and Slovakia. Sustainability 10,
570 1311.

571 Olmstead, S., 2019. Pollinators and Pollination of Haskap (Lonicera caerulea L.) in Southern
572 Nova Scotia.

573 Ottosen, C.-O., 1987. Male bumblebees (Bombus hortorum l.) as pollinators of Lonicera
574 periclymenum L. in NE-Zealand, Denmark. Flora 179, 155–161.

575 Perfecto, I., Vandermeer, J., 2010. The agroecological matrix as alternative to the land-
576 sparing/agriculture intensification model. Proceedings of the National Academy of Sciences
577 107, 5786–5791.

578 Petersen, J.D., Reiners, S., Nault, B.A., 2013. Pollination services provided by bees in
579 pumpkin fields supplemented with either Apis mellifera or Bombus impatiens or not
580 supplemented. PLoS One 8, e69819.

581 Phalan, B., Onial, M., Balmford, A., Green, R.E., 2011. Reconciling food production and
582 biodiversity conservation: land sharing and land sparing compared. Science 333, 1289–1291.

583 Racys, J., Montviliene, R., 2005. Effect of bees-pollinators in buckwheat [Fagopyrum
584 esculentum Moench] crops. Journal of Apicultural Science 49.

585 Rader, R., Howlett, B.G., Cunningham, S.A., Westcott, D.A., Newstrom-Lloyd, L.E., Walker,
586 M.K., Teulon, D.A., Edwards, W., 2009. Alternative pollinator taxa are equally efficient but
587 not as effective as the honeybee in a mass flowering crop. Journal of Applied Ecology 46,
588 1080–1087.

589 Řezníček, V., Salaš, P., 2004. Gene pool of less widely spread fruit tree species. Acta
590 Universitatis Agriculturae et Silviculturae Mendelianae Brunensis 52, 159–168.

591 Stanley, D.A., Gunning, D., Stout, J.C., 2013. Pollinators and pollination of oilseed rape crops
592 (Brassica napus L.) in Ireland: ecological and economic incentives for pollinator conservation.
593 Journal of Insect Conservation 17, 1181–1189.

594 Stoate, C., Boatman, N., Borralho, R., Carvalho, C.R., De Snoo, G., Eden, P., 2001.
595 Ecological impacts of arable intensification in Europe. Journal of environmental management
596 63, 337–365.

597 Svarcovaa, I., Heinrichb, J., Valentovaa, K., 2007. Berry fruits as a source of biologically
598 active compounds: the case of Lonicera caerulea. Biomedical Papers of the Medical Faculty
599 of Palacky University in Olomouc 151.

600 ter Braak, C.J., Schaffers, A.P., 2004. Co-correspondence analysis: a new ordination method
601 to relate two community compositions. Ecology 85, 834–846.

602 Upton, M., Mantle, B., Hastings, A., 2010. Methods for collecting, preserving and studying
603 insects and other terrestrial arthropods. Australian Entomological Society.

Von Der Ohe, W., Oddo, L.P., Piana, M.L., Morlot, M., Martin, P., 2004. Harmonized methods of melissopalynology. Apidologie 35, S18–S25.

Williams, I.H., 1985. The pollination of swede rape (Brassica napus L.). Bee World 66, 16–22.

Woodcock, B., Edwards, M., Redhead, J., Meek, W., Nuttall, P., Falk, S., Nowakowski, M., Pywell, R., 2013. Crop flower visitation by honeybees, bumblebees and solitary bees: Behavioural differences and diversity responses to landscape. Agriculture, Ecosystems & Environment 171, 1–8.

Żebrowska, J., 1998. Influence of pollination modes on yield components in strawberry (Fragaria× ananassa Duch.). Plant Breeding 117, 255–260.

Diversity of bees (Apoidea) and their pesticide contamination in two different types of agricultural management

Marian Hybl[1], Petr Mraz[2], Jan Sipos[1]
[1]Department of Zoology, Fisheries, Hydrobiology and Apidology
Mendel University in Brno
Zemedelska 1, 613 00 Brno
[2]Department of Plant Production
University of South Bohemia in Ceske Budejovice
Studentska 1668, 370 05 Ceske Budejovice
CZECH REPUBLIC

Mario.eko@seznam.cz

Abstract: The biodiversity and density of pollinators systematically and drastically decreases. The intensification of agriculture, including a change in land use, increased chemistry and different farm management are considered to be the main causes. In the study differences in land use, biodiversity of apidofauna and chemical contamination of bees by pesticides was compared between the locations with different management. While the locality in conventional regime of agriculture was characterized by intensively farmed rapeseed field and main landscape matrix was formed by arable land, at the locality in organic regime of agriculture was observed flowering strips and major landscape matrix was formed by permanent grassland. At the locality in organic regime, a significantly higher richness and abundance of bees were observed in comparison with the locality in conventional regime. In addition, the analyses of bees from the locality in conventional regime revealed the presence of residues of several pesticides. In contrast, at the locality in organic regime, analyses did not confirm presence of any residues.

Key Words: biodiversity, pesticides, pollinators, contamination

INTRODUCTION

Apoidea are a diverse group comprising solitary, socially or kleptoparasitic species living exclusively on pollen and nectar (Macek et al. 2010). The bee superfamily includes approximately 430 genera and about 16,000 species. The most important agricultural genera in the Czech Republic includes bees (Apis), bumblebees (Bombus) and solitary bees, of which there are 6 genera and about 600 species in the Czech Republic (Straka et al. 2010). Bees are the most important pollinators of agricultural crops and it is estimated that 30% of crop production depends on bee pollination (O'Toole 1993). In addition, many wild plants (estimated at 60–90%) are dependent on insect pollination, with bees making the greatest contribution to pollination (Spivak et al. 2011). Except to improving the qualitative and quantitative characteristics of agricultural production (especially vegetables, fruits and seeds) (Vládek et al. 2018), bee activity also contributes significantly to wider plant biodiversity and improved ecosystem equilibrium (Delaphane and Mayer 2000).

Thus, the decline in the diversity of wild pollinators and the massive collapses of *A. mellifera* colonies are extremely worrying (Biesmeijer et al. 2006). This pollinator crisis is expected to continue to have a far-reaching impact not only on agriculture and the related economy (Gallai et al. 2009), but also on the species diversity of plants associated with them (Biesmeijer et al. 2006) and the overall landscape character (Ricketts et al., 2008). The main factors contributing to the decline of native pollinator species are landscape fragmentation, habitat loss, pesticide use (Cane and Tepedino 2001). In addition to declining wild bee populations in recent years, there have also been severe losses of *A. mellifera* colonies due to pests, diseases and especially pesticide pollution and malnutrition (Matheson et al. 1996, Hýbl et al. 2019).

Pesticide pollution in intensively farmed landscapes is a dangerous phenomenon because these substances accumulate in vegetation, water and soil and cause damage to beneficial organisms such

as bees (Porrini et al. 2002). In agricultural areas, *A. mellifera* colonies are commonly used as bioindicators of environmental pollution. In addition to the state of the colony, chemical pollution of the environment is detected by chemical analyses of bees and bee products, which tells us about the state of the environment. This makes bees a generally accepted bioindicator (Balayiannis and Balayiannis 2008).

The aim of the study is to verify the influence of the intensity of agricultural management on the population of bees (Apoidea), to describe the differences in diversity, abundance, balance and contamination of the bee community in differently managed agricultural localities.

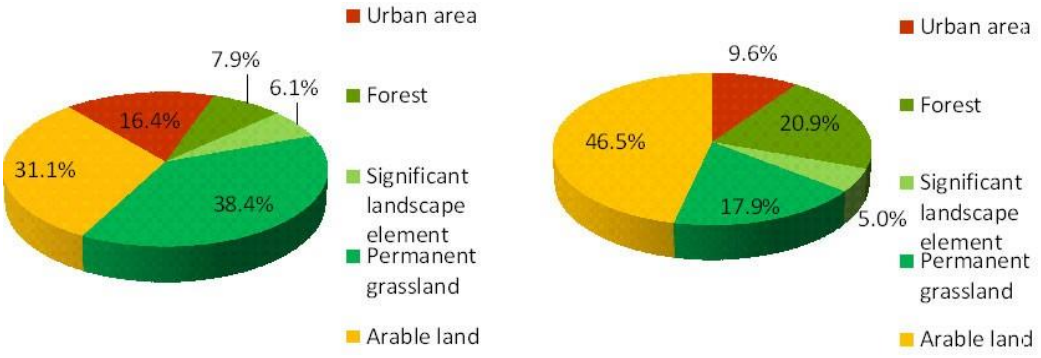
MATERIAL AND METHODS

At both localities, measuring of biodiversity and abundance were done and chemical analyses of bodies of bees (*A. mellifera*) caught at the entrance of hives located in close proximity to the localities was performed.

Study areas

The locality in the ecological regime of agriculture was located near the village of Malonty. The landscape matrix consisted mainly of permanent grasslands, which were also exclusively in the ecological regime of farming. In addition to permanent grassland, there were a large number of significant landscape elements and an inconsiderable part of landscape was occupied by forests. However, the observation and collection of bees took place in flowery belts dividing the field, which was also in mode of organic agriculture. A large part of the landscape was occupied by the development of the village (Figure 1). The average slope of the site is 5 °, the average altitude is 680 meters. In recent years, in addition to cereals, a significant number of clovers have been grown in the locality and its surroundings, and flowering catch crops and buckwheat are also an integral part of sowing procedures, which provide bees with a valuable source of food. The location in the conventional agricultural regime was located near the town of České Budějovice. The landscape matrix consisted mainly of arable land, thus intensively cultivated areas on which most oilseed rape was grown. However, in the immediate vicinity of the monitored locality, there were also more extensively used areas, mainly regularly mown grasslands and commercial forests. There were also several other ecologically important areas in the landscape (borders, draws, tree lines and others). Apart from that, the urban area is also represented to a lesser extent (Figure 1). The monitored field had an area of 1.83 ha, a slope of 2 ° and an average altitude of 520 meters. In recent years, almost only rape, corn, and wheat have been grown in the area.

Figure 1 Graph of land use in radius 1km in observed localities (locality in organic regime in left, locality in conventional regime in right)



Sampling

Measurement of bee diversity and abundance was based on trapping in the Moericke traps. The principle of the traps lies in the attractiveness of the yellow colour for the bees, which drown after sitting on the surface. Traps were collected regularly every 14 days. Bee diversity was measured throughout the flowering period of rapeseed at the locality in conventional regime. This period also coincided with the flowering of the main blossoming species at the locality in organic regime.

The material was then determined and classified into families and species. Based on this, the Shannon-Weaver diversity index and equitability were calculated.

Analyses of pesticides

The workers of western honey bee (*A. mellifera*) used for pesticide contamination analyses were obtained by capture at a time when the last chemical treatment at rapeseed field had already been provided. On May 9, approximately 1,000 *A. mellifera* individuals were captured at the site in the conventional agricultural regime, and approximately 1,000 *A. mellifera* individuals were also captured at locality in organic farming regime on May 10. The analyses of contamination of bees were performed by the State Research Institute in Prague. Methods used: SOP 10.58 (GC-ECD) and SOP 70.101 (HPLC-MS / MS). Using these methods, the presence and amount of pesticides and their residues were detected.

Statistical analyses

Individual study plots were clustered within fields and each plot was repeatedly sampled over time. Accordingly, due to nested design, the mixed effect model procedure was applied to data analysis. The relationship between species richness and abundance as dependent variables and the agriculture management as explanatory variable was separately evaluated by two linear mixed effect models (LMMs) with Poisson error distribution and link function log. In the LMM model, particular plot was used as random intercept effect and sample date as random slope effect. The significance of the explanatory variables was then tested by Likelihood Ratio Test.

RESULTS

During the monitoring, a total of 310 individuals of bees belonging to 5 families representing 39 species of bees were observed (Figure 3 and 4). At the locality in organic regime, altogether 36 species of bees from 5 families (Apidae, Andrenidae, Colletidae, Halictidae and Megachilidae) were observed. The most abundant group in terms of species and numbers were bees of the family Andrenidae, which numbered 17 species, 147 individuals and accounted for 64.2% of all observed bees. The most abundant species were *Andreana cineraria* (76 individuals, 33.2%), *Andrena Bicolor* (30 individuals, 13.1%) and *A. mellifera* (27 individuals, 11.8%). However, compared to the abundance of solitary bees, a small occurrence of bumblebees was found, including 2 species *of Bombus terrestris* (4 individuals, 1.7%) and *Bombus lucorum* (1 individual, 0.4%). The least numerous family was Megachilidaee represented by 2 species of bees, *Chelostoma florisomne* numbered only one individual, *Chelostoma rapunculi* numbered 3, which together accounted for 1.7% of the total number of captured individuals. In the locality in convetional regime were observed 20 species of bees from 3 families (Apidae, Andrenidae and Halictidae) The most abundant group in terms of species and numbers were bees from the Andrenidae family, which numbered 13 species, 50 individuals that accounted for 61.7% of all caught bees. However, the most abundant species was *A. mellifera* of the genus Apidae, which represented 15 individuals and 18.5% of all bees collected. A significant proportion of bumblebees were also found. Two species were captured, numbering a total of 10 individuals, most of which were *B. Terrestris* and a smaller part of *Bombus lapidarius*, together accounting for 12.3%. The least numerous family was Haliactidae in which, despite the presence of 4 species, only 6 individuals were caught, which gave only 7.5% of the total number. The graphs show a difference in the composition of the pollinator community in the both localities. In the ratio of occurrence and richness of species, localities vary considerably. While the locality in organic agriculture has 5 families representing 36 species, the locality of conventional agriculture has only 3 families including only 20 species. This corresponds to the value of Shannon-Weaver index and equitability, which is about twice as high in the locality in organic regime, compared to the locality in conventional regime. Similarly, in the case of the locality in organic regime, the species richness (χ^2= 4.5717, p = 0.0325) and species abundance (χ^2 = 13.313, p < 0.001) are significantly higher compared to the locality in conventional regime (Figure 4).

The most significant difference between localities was in the presence of pesticides. At the locality in convenctional agriculture, altogether 4 pesticides were detected (Table 2), all of them correspond with pesticides applied on the rapeseed field (Table 1), except for carbendazim (which is a metabolite of thiophanate methyl). In the other hand, at the locality in organic regime, no pesticides were found.

Figure 2 Comparison of the percentage of bee families between different farming systems of over time

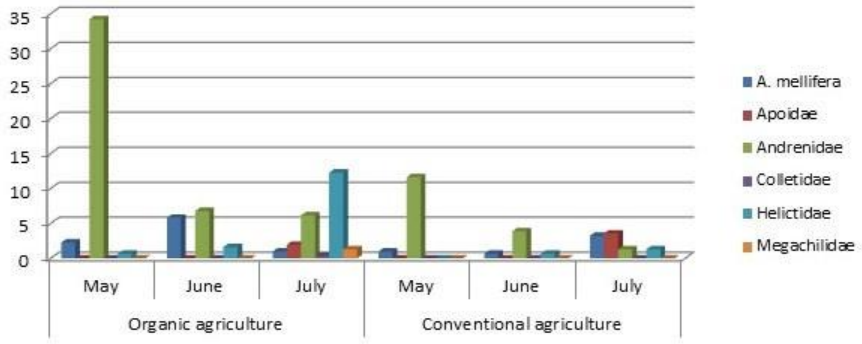


Figure 3 Comparison of species and families richness between different farming systems (in left) and value of Shannon-Weaver index and equitability (in right)

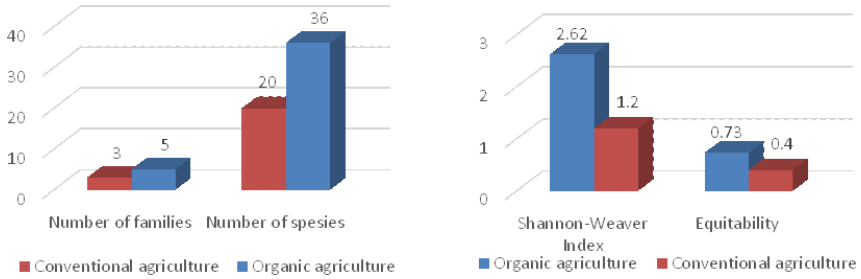


Figure 4 Comparison of species abundance (in left) and species richness (in right) between different farming systems

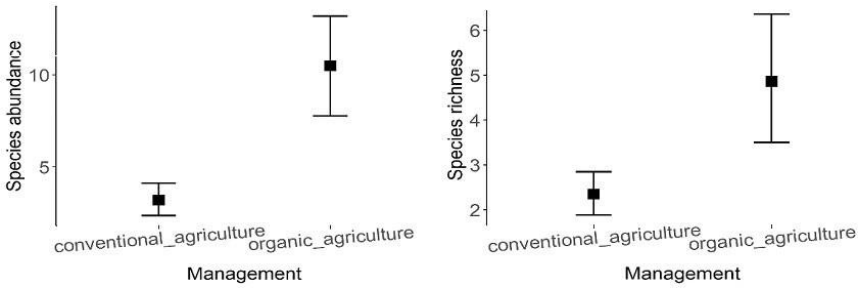


Table 1 Pesticides used on rapeseed field at the locality in the conventional regime

Trade name	Date of application	Amount	Active substances	Function
Succesor 600	21. 08. 2015	1.83 l/ha	pethoxamid 600 g/l	herbicide
Command 36CS	21. 08. 2015	0.145 l/ha	clomazone 360 g/l	herbicide
Groundet	21. 08. 2015	0.3 l/ha	732 g/l paraffin oil	wetting agent
Metarex	26. 08. 2015	4 kg/ha	metaldehyde 40 g/kg	molluscicide
Garland forte	1. 09. 2015	0.6 l/ha	propaquizafop 100 g/l	herbicide
Lynx	2. 10. 2015	0.95 l/ha	tebuconazole 250h/l	fungicide
Nurelle D	4. 4. 2016	0.6 l/ha	chlorpyrifos 500 g/l cypermethrin 50 g/l	insecticide
Bariard	2. 5. 2016	0.3 l/ha	thiacloprid 240 g/l	insecticide
Paroli	2. 5. 2016	3 l/ha	thiophanate methyl 167 g/l iprodione 167 g/l	fungicide

Table 2 Detected pesticides in bee bodies captured at the locality in conventional regime of agriculture

Pesticide	Amount (mg/kg)	Function	Toxicity	LD50 bee	Detected amount per 1 bee
thiophanate-methyl	0.119 (± 50 %)	fungicide	slight	>100 µg	0.0119µg
thiacloprid	0.114 (± 50 %)	insecticide	low	24 µg	0.0114µg
iprodione	0.084 (± 50 %)	fungicide	slight	>100 µg	0.0084µg
carbendazim	0.067 (± 50 %)	fungicide	low	50 µg	0.0067µg

DISCUSSION

When comparing the localities, there is a clear difference in the composition of the pollinator community. In the ratio of occurrence and richness of species, the localities differ considerably (Figure 3 and 4). Higher species diversity and abundance of bees on the locality in the organic regime of agriculture was probably caused by lower farming intensity, i.e., lower environmental chemistry, but higher landscape heterogenity and higher diversity of cultivated plants, which is in agreement with literature (Cane and Tepedino 2001, Porrini et al. 2002). This suggests that the ecological management system is a much more suitable environment for apidofauna compared to the conventional way of management, represented by the locality near České Budějovice. The results of the Shannon-Weaver index and equitability point to a markedly higher equilibrium and stability of the pollinator community in space and time (which is also evident from Figures 2 and 3). However, the higher proportion of bumblebees (Bombus spp.) and *A. mellifera* in the locality in the conventional regime points the ability of generalist bee species to some extent adapt to the anthropogenic environment to take the place of more specialized pollinators and thus replace their pollination service, which is in accordance with literature (Ghazoul 2005).

Although the determined amount of pesticide residues in bee bodies was very low, a negative effect on bee populations cannot be ruled out (Blackuiére 2012), which can be difficult to detect at such low doses (Lambin et al. 2001). Another potential risk, even with such low doses of pesticides, is a possible synergistic effect (Iwasa et al. 2004). In addition to direct toxicity, persistent exposure to pesticides at low doses can disrupt the bees' immune system, making it more susceptible to viral infections to which bees are normally resistant (Di Prisco 2013). Last but not least, the question remains as to the mortality of bees contaminated with a lethal dose and what was the concentration of pesticides in such affected individuals. The presence of natural and semi-natural habitats (Carré et al. 2009) certainly has another effect on the differences in diversity of pollinators in monitored localities, which is again related to the degree of landscape intensification.

CONCLUSION

The locality in the regime of conventional agriculture was characterized by a lower richness and abundance of bees. Furthermore, a lower number of specialists and, conversely, a higher number of generalists were observed in comparison with the locality in the ecological regime of agriculture.

The bee population at the site in the conventional regime was contaminated with pesticides. Residues of several pesticides were detected directly in bodies of bees, in contrast, in bees from the locality in the regime of organic farming, no pesticide residues were detected.

ACKNOWLEDGEMENTS

We are indebted to Martin Šlachta for help with determination of bees and Jiří Peterka (both from University of South Bohemia in České Budějovice) for help with collection of samples.

REFERENCES

Balayiannis, G., Balayiannis, P. 2008. Bee honey as an environmental bioindicator of pesticides occurrence in six agricultural areas of Greece. Archives of Environmental Contamination and Toxicology, 55(3): 462–470.

Biesmeijer, J.C. et al. 2006. Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. Science, 313(5785): 351–354.

Blacquiére, T. et al. 2012. Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. Ecotoxicology, 21: 973–992.

Cane, J.H., Tepedino, V.J. 2001. Causes and extent of declines among native North American invertebrate pollinators: detection, evidence, and consequences. Conservation Ecology, 5(1): 1.

Carré, G. et al. 2009. Landscape context and habitat type as drivers of bee diversity in European annual crops. Agriculture, Ecosystems and Environment, 133(1–2): 40–47.

Delaphane, K.S., Mayer, D.R. 2000. Crop Pollination by Bees. 1st ed., Washington State University, USA: Cabi Publishing.

Di Prisco, G. et al. 2013. "Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees". Proceedings of the National Academy of Sciences, 110(46): 18466–18471.

Gallai, N. et al. 2009. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecological Economics, 68(3): 810–821.

Ghazoul, J. 2005. Buzziness as usual? Questioning the global pollination crisis. Trends in Ecology and Evolution, 20(7): 367–373.

Hýbl, M. et al. 2019. Effects of phenolic bioactive substances on reducing mortality of bees (*Apis mellifera*) intoxicated by thiacloprid. In Proceedings of International PhD Students Conference, MendelNet 2019 [Online]. Brno, Czech Republic, 6–7 November, Brno: Mendel University in Brno, Faculty of AgriSciences, pp. 131–135. Available at: https://mendelnet.cz/pdfs/mnt/2019/01/21.pdf [2020-09-16].

Iwasa, T. et al. 2004. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, Apis mellifera. Crop Protection, 23(5): 371–378.

Lambin, M. et al. 2001. Imidacloprid-induced facilitation of the proboscis extension reflex habituation in the honeybee. Archives of Insect Biochemistry and Physiology, 48: 129–134.

Macek, J. 2010. Blanokřídlí České republiky I. - Žahadloví. 1. Vyd., Praha: Academia. Atlas (Academia).

O`Toole, C. et al. 1993. Diversity of native bees and agroecosystems. In Hymenoptera and biodiversity. Wallingford: CAB International, pp. 169–196.

Porrini, C. et al. 2014. Using honey bee as bioindicator of chemicals in Campanian agroecosystems (South Italy). Bulletin of Insectology, 67(1): 137–146.

Spivak, M. 2011. The Plight of the Bees. Environmental Science and Technology, 45(1): 34–38.

Straka, J. et al. 2007. Apoidea: Apiformes (včely). Acta Entomologica Musei Nationalis Pragae, 11: 241–299.

Vládek, A. et al. 2018. Pollination and pollinators of Hascap (Lonicera caeruela). In Proceedings of International PhD Students Conference MendelNet 2018 [Online]. Brno, Czech Republic, 7–8 November, Brno: Mendel University in Brno, Faculty of AgriSciences, pp. 211–216. Available at: http://mendelnet.cz/pdfs/mnt/2018/01/43.pdf. [2020-09-16].

insects

MDPI

Article

Polyphenols as Food Supplement Improved Food Consumption and Longevity of Honey Bees (*Apis mellifera*) Intoxicated by Pesticide Thiacloprid

Marian Hýbl [1], Petr Mráz [2], Jan Šipoš [1], Irena Hoštičková [2], Andrea Bohatá [3], Vladislav Čurn [2] and Tomáš Kopec [4,*]

1 Department of Zoology, Fisheries, Hydrobiology and Apiculture, Faculty of AgriSciences, Mendel University in Brno, Zemedelska 1, 613 00 Brno, Czech Republic; mario.eko@seznam.cz (M.H.); jan.sipos@mendelu.cz (J.Š.)
2 Department of Genetics and Agricultural Biotechnology, Faculty of Agriculture, University of South Bohemia in Ceske Budejovice, Studentska 1668, 370 05 Ceske Budejovice, Czech Republic; mrazpe01@zf.jcu.cz (P.M.); jelini00@zf.jcu.cz (I.H.); curn@zf.jcu.cz (V.C .)
3 Department of Crop Production, Faculty of Agriculture, University of South Bohemia in Ceske Budejovice, Studentska 1668, 370 05 Ceske Budejovice, Czech Republic; bohata@zf.jcu.cz
4 Department of Animal Breeding, Faculty of AgriSciences, Mendel University in Brno, Zemedelska 1, 613 00 Brno, Czech Republic
* Correspondence: tomas.kopec@mendelu.cz

Simple Summary: Worldwide, mass losses of honey bee colonies are being observed more frequently. Poor nutrition may cause honey bees to be more susceptible to pesticides and more vulnerable to diseases, and as a direct result of this, honey bee colonies can collapse. Another cause of mass bee colony collapse that is no less important is the use of pesticides. The level of toxicity of most pesticides is greatly affected by nutrient uptake. In addition, the honey bee genome is known to be specific for a significantly lower number of genes associated with detoxification compared with other insect species. Intake of phenolic and flavonoid substances in food can lead to increased expression of genes encoding detoxification enzymes in bees. Therefore, in this study, we evaluated in vitro the effect of phenolic and flavonoid substances on bee mortality and food consumption in the case of intoxication by pesticide thiacloprid. The results of this study showed a significant positive effect on honey bee survival rate as well as increased food intake. In addition, the expression level of genes encoding detoxification enzymes was determined.

Abstract: Malnutrition is one of the main problems related to the global mass collapse of honey bee colonies, because in honey bees, malnutrition is associated with deterioration of the immune system and increased pesticide susceptibility. Another important cause of mass bee colonies losses is the use of pesticides. Therefore, the goal of this study was to verify the influence of polyphenols on longevity, food consumption, and cytochrome P450 gene expression in worker bees intoxicated by thiacloprid. The tests were carried out in vitro under artificial conditions (caged bees). A conclusively lower mortality rate and, in parallel, a higher average food intake, were observed in intoxicated bees treated using a mixture of phenolic acids and flavonoids compared to untreated intoxicated bees. This was probably caused by increased detoxification capacity caused by increased expression level of genes encoding the cytochrome P450 enzyme in the bees. Therefore, the addition of polyphenols into bee nutrition is probably able to positively affect the detoxification capacity of bees, which is often reduced by the impact of malnutrition resulting from degradation of the environment and common beekeeping management.

Keywords: cage experiments; cytochrome P450; detoxification; food intake; mortality rate

Citation: Hýbl, M.; Mráz, P.; Šipoš, J.; Hoštičková, I.; Bohatá, A.; Čurn, V.; Kopec, T. Polyphenols as Food Supplement Improved Food Consumption and Longevity of Honey Bees (*Apis mellifera*) Intoxicated by Pesticide Thiacloprid. *Insects* **2021**, *12*, 572. https://doi.org/10.3390/insects12070572

Academic Editors: Ivana Tlak Gajger and Franco Mutinelli

Received: 19 May 2021
Accepted: 16 June 2021
Published: 23 June 2021

1. Introduction

One of the most worrying phenomena is the global mass losses of honey bee colonies, including in Europe and the USA [1,2]. Along with diseases, nutrition stress and malnutrition appears to be one of the main causes of bee mortality [3–5]. The healthy development and survival of bee colonies depends to a large extent on the availability and quality of nutrients in the environment [3,6]. However, the availability and diversity of bee food resources are steadily declining due to the ever-increasing intensification of agriculture and the associated changes in the landscape, leading to a decrease in environmental sustainability [7]. As a result, there has been a decrease in the diversity of flowering plants, and low species diversity of blooming plants means reduced availability and diversity of macro- and microelements in bee nutrition [4,8], which, in the end, negatively affects bee populations [7,9]. The lack of nutrients is also the result of inefficient beekeeping practices; when replenishing winter supplies, bees are often provided only with a solution of sugar and artificial pollen substitutes. These food supplements usually lack nutrients that are naturally occurring in bees' natural diets [10]. Consequently, bee colonies are not provided with full-value nutrition [11]. Poor nutrition may cause greater susceptibility to pesticides [12], more vulnerability to diseases [13], and, as a direct result of this, the number of honey bee colonies may be decreasing [14].

Another cause of mass bee colony collapse that is no less important is the use of pesticides [15,16], which can act synergistically with other pesticides [17] or with pathogens [18,19]. The level of toxicity of most pesticides varies depending on many factors, including the means of exposure, the age of the bees, the fitness of the colonies or bee subspecies [20,21], and the optimal nutrient distribution [4,22]. In addition, the degree of toxicity of different pesticides may vary depending on whether they are tested on individual bees or on whole colonies. In vitro tests often show high pesticide toxicity and associated negative effects on bees [17,18,23]; in contrast, entire colonies appear to be relatively less susceptible to pesticides [24]. A similar trend can be observed in other social bees such as bumblebees [25]. In a broader context, some bee species may even be advantaged in anthropogenic areas such as agricultural land or urban areas [26]. However, the results of the study by Alburaka et al. [27] suggest that, while neonicotinoids do not directly affect the health and strength of bee colonies, they indirectly weaken bee health by inducing physiological stress and increasing the burden of pathogens.

However, the bee genome is known to be specific for a significantly lower number of genes associated with detoxification compared to other insect species. Where the honey bee has only 46 genes encoding the cytochrome P450 enzyme, which is thought to be the major enzyme responsible for detoxification, other insect species have around 80 or more genes encoding the cytochrome P450 enzyme [28]. There are several honey bee cytochrome P450 genes that have defined functions, including CYP9Q1, CYP9Q2, and CYP9Q3. These genes metabolize both natural and synthetic xenobiotics [29].

The intake of phenolic and flavonoid substances, which are commonly found in honey and, to a greater extent, in pollen, via food can lead to an increased expression of genes encoding cytochrome P450 enzyme in bees. The amount and proportion can vary significantly depending on food sources [30]. Of these, the highest efficacies have been observed for p-coumaric acid and quercetin [31]. The natural diet of bees usually contains a large amount and great diversity of phenolic acids, flavonoids and their derivatives [32,33] and it is their different amounts and proportions that influence the detoxifying effects [31].

The first goal of this study was to determine the real effect of phenolic acids and flavonoids in vitro on the mortality of bees intoxicated by thiacloprid, one of the most widely used neonicotinoids. The second aim was to determine the effect of phenolic substances on the rate of food intake by bees; and the last target, although no less important, was to determine the expression level of several genes potentially responsible for detoxification via the enzyme cytochrome P450.

2. Materials and Methods

The experiment was carried out at the beginning of the summer of 2019 in Brno (South Moravia, Czech Republic)

2.1. Bees

The honey bees used in this study were obtained from the experimental apiary of Mendel University in Brno. Honey bees from four colonies were used (one frame with hatching bees per colony). The colonies were maintained following standard beekeeping practices. In all the bee colonies, inseminated queens belonging to *Apis mellifera carnica* were used. As a result, the genetic variability of bees in individual colonies was reduced so that the average coefficient of relatedness between workers from one colony was r = 0.5.

The brood frames with hatching bees (one from each colony) were incubated at 35 ◦C and 65–80% relative humidity for 12 h. This allowed bees of the same age ± 12 h to be obtained. Then the frames were brushed, and all bees were mixed together and divided into four groups according to the treatment with three replications (three cages each). There were 40 bees of the same age in each cage. The cages were maintained for 2 weeks in the thermostat with conditions 30 ◦C and 65–70% relative humidity [34]. The bee mortality and food consumption were noted down every day and dead bees were continuously removed from the cages.

2.2. Chemicals

The sucrose solution consisted of 50% (*w/v*) sucrose and distilled water. A dosage of thiacloprid was mixed with the sucrose solution in two different concentrations, 35 mL/L or 70 mg/L, depending on the treatment [19].

The mixture of phenolic compounds consisted of 200 mg/kg of phenolic acids and 10 mg/kg of flavonoids in proportions based on the real concentrations found in common honey [33]. The concentration of p-Coumaric acid was scaled up on the basis of Mao et al. [30]. The final contain of phenolic compounds is in Table 1. The thiacloprid and sucrose were purchased from Sigma Aldrich (Schnelldorf, BO, Germany), phenolic acids and flavonoids were purchased from Alfa Aestar (Kandel, RP, Germany).

Table 1. Content of phenolic acids and flavonoids used in the phenolic mixture.

Phenolic Substance Classification	Phenolic Substance Name	Amount (%)	Amount (mg/kg)
Phenolic acids	caffeic acid	10	20
	benzoic acid	20	40
	gallic acid	7.5	15
	ferulic acid	20	40
	p-Coumaric acid	35	70
	vanillic acid	7.5	15
Flavonoids	rutin	25	2.5
	quercetin	25	2.5
	naringin	25	2.5
	hesperidin	25	2.5

2.3. Design of the Experiment

The bees in cages were fed with two top feeders with scales (*ad libitum*) per cage, enabling measurement of the daily food consumption. The rate of consumption of a prequantified amount by a set number of live bees was evaluated over a set time period. The experimental groups were set up as follows:

1. Treatment TL—sucrose solution with a low dosage of Thiacloprid (35 mg/L).

2. Treatment FTL—sucrose solution (50% *w/v*) with a mixture of phenolic compounds and low dosage of Thiacloprid (35 mg/L).
3. Treatment TH—sucrose solution (50% *w/v*) with high dosage of Thiacloprid (70 mg/L).
4. Treatment FTH—sucrose solution (50% *w/v*) with a mixture of phenolic compounds and a high dosage of Thiacloprid (70 mg/L).
5. Treatment F—sucrose solution (50% *w/v*) and a mixture of phenolic compounds.
6. Treatment C—sucrose solution (50% *w/v*).

2.4. RNA Isolation and RT-qPCR

Samples for studying gene expression were collected as a bulk of three bees and frozen immediately in liquid nitrogen, and were stored at −80 ◦C. Total RNA was extracted using the TRI Reagent (MRC, Montgomery, OH, USA) according to manufacturer's instructions. Contaminating DNA was removed using the DNA-freeTMKit (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 stored at −20 ◦C until use.

The RT-qPCR 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 three replicates and no-template controls were included. The specificity of amplification was determined by dissociation curve analyses. A comparative threshold cycle method was applied to determine relative concentrations of mRNA. The primers used are shown in Table 2. All the gene expression levels were normalized to Am Rp49 gene expression, as a reference gene [35], and the obtained data were normalized to Am Rp49 using the ΔΔCT method according to Livak, Schmittgen [36].

Table 2. Primers for qPCR analysis.

Gene	Sequences 5′-3′	Reference
Cyp9q1	F: TCGAGAAGTTTTTCCACCG R: CTCTTTCCTCCTCGATTG	Mao et al. [37]
Cyp9q2	F: GATTATCGCCTATTATTA R: GTTCTCCTTCCCTCTGAT	Mao et al. [37]
Cyp4g11	F: AATGCGAGAAGTGTCGTCGA R: AGCGGTTTCCAGAAGGATGT	Calla et al. [38]
AmRp49	F: CGTCATATGTTGCCAACTGGT R: TTGAGCACGTTCAACAATGG	Tesovnik et al. [39]

2.5. Data Analyses

The survival curves were fitted by the Kaplan-Meier method. On the basis of this method, the survival probability for each tested treatment during 14 days of observation was estimated [40]. The conclusive difference between each survival curve was evaluated by log-rank test [41]. The log-rank test compares a monitored case number with the case number that would have been expected under the null hypothesis (i.e., identical survival curves). All data were analyzed using the R statistical program (R Core Team, 2017).

Daily food intake was analyzed using the statistical program Statistica 12. The effect of fed substances on the rate of diet consumption was tested by the analysis of variance procedure ANOVA (post hoc analysis using Tukey test), preceded by a normality test. Statistical significance was tested at a level of significance $\alpha = 0.05$.

3. Results

The bee survival rate corresponding fed treatment is presented in Table 3 and Figure 1. The FTH group exhibited a significantly lower mortality rate than group TH ($p < 0.001$), but a higher mortality rate than the control groups C and F ($p < 0.001$ for both). Comparatively lower mortality rates were observed in the treatment FTL than in the treatment TL ($p < 0.001$), although the mortality rate was higher than in the control group C ($p = 0.03$), but no significant differences were observed in comparison with group F ($p = 0.17$). Additionally, no significant differences were registered between control groups C and F ($p = 0.44$). Significant differences were also observed between TH and C, TH and F, TL and C, and TL and F ($p < 0.001$ in all cases).

Table 3. The results of the log-rank test, which was used to compare different treatment groups of bees treated using various chemical substances.

Treatment	Degrees of Freedom	Chi-Square Statistic	*p*-Value
TH/C	1	310	<0.001
TH/F	1	270	<0.001
FTH/TH	1	72	<0.001
FTH/C	1	62.9	<0.001
FTH/F	1	51.2	<0.001
FTL/TL	1	6	0.01
TL/C	1	5.7	0.01
FTL/C	1	4.6	0.03
FTL/F	1	1.8	0.17
F/C	1	0.6	0.44

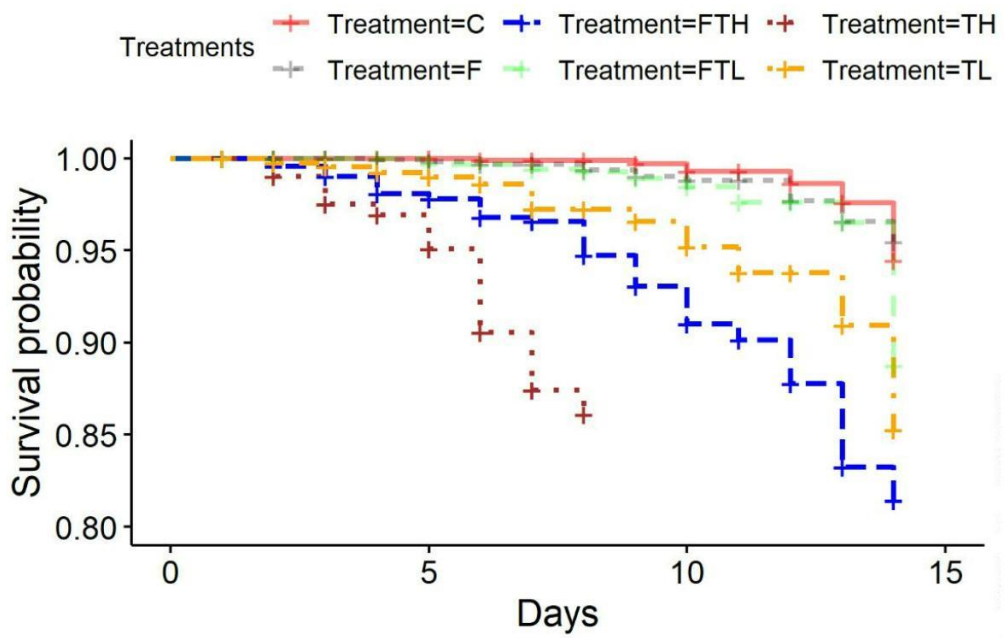


Figure 1. The relationship between the bee mortality and the treatments over 14 days (Kaplan-Maier survival analyses). Legend: TH—sucrose solution (50% *w/v*) with high dosage of Thiacloprid (70 mg/L), FTH—sucrose solution (50% *w/v*) with a mixture of phenolic compounds and a high dosage of Thiacloprid (70 mg/L), TL—sucrose solution with a low dosage of Thiacloprid (35 mg/L), FTL—sucrose solution (50 *w/v*) with a mixture of phenolic compounds and low dosage of Thiacloprid (35 mg/L), F—sucrose solution (50% *w/v*) and a mixture of phenolic compounds, C—sucrose solution (50% *w/v*).

The food intake was dependent on the treatment (Figure 2). The amount of diet consumed was higher in groups C and F than in any of the other groups, whereas the food

consumption was higher in group F than in group C. In group FTL, higher food intake was observed than in group TL. The same trend was observed in the case of the FTH and TH groups. The lowest food consumption was observed in groups TH and TL.

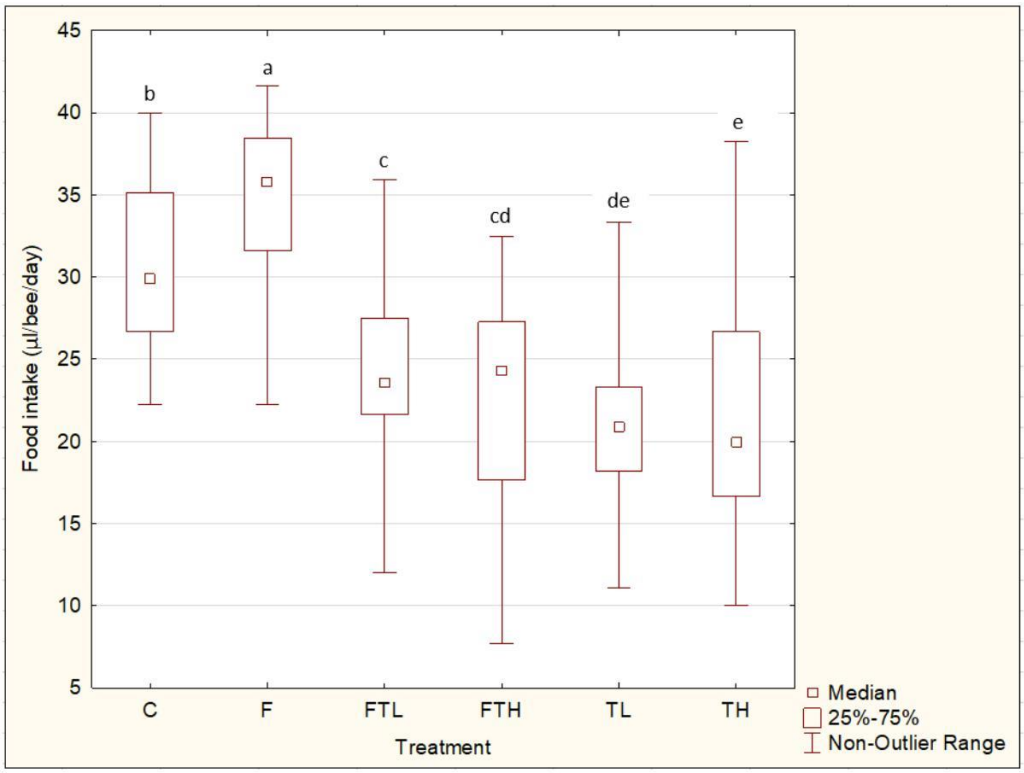


Figure 2. The daily average of the food intake per 1 bee over 14 days. Significant differences ($p < 0.05$) in food consumption between treatments are indicated by different letters.

The expression of CYP9Q1 (Figure 3a), CYP9Q2 (Figure 3b), CYP9Q3 (Figure 3c) and CYP4G11 (Figure 3d) genes was analyzed using RT-qPCR. Differences in gene expression between the testing groups were not statistically significant. However, despite that, some trends in the levels of expression were noted between the testing groups. The relative expression of the CYP9Q1 gene decreased in bees fed with sucrose solution enriched by phenolic compounds, irrespective of thiacloprid intoxification (F, FTL, FTH) in comparison with the C group after 7 days of treatment. In bees from groups TL and TH, the relative gene expression of this gene was comparable with its expression in the C group after 7 days. After 14 days of treatment, the relative expression of CYP9Q1 was increased in bees from group TH. In other groups, the relative gene expression of this gene was comparable with its expression in the C group.

After 7 days of treatment, the relative expression of CYP9Q2 in bees from groups F and FTL was comparable with the C group. In groups FTH, TL and TH, it was slightly increased in comparison with the C group. After 14 days of treatment, the relative expression of this gene was increased in groups F, FTL and FTH. In groups TH and TL it was comparable with group C.

The relative expression of CYP9Q3 was higher in the TL group after 7 days of treatment and also in the FTH and TL groups after 14 days of treatment. In other groups, the relative expression of this gene was comparable with group C.

After 7 days of treatment, the relative expression of the CYP4G11 gene was comparable in bees fed with sucrose solution enriched by phenolic compounds regardless of whether they were intoxicated with thiacloprid (F, FTL, FTH) and in bees from C group. In groups TL and TH, it was increased in comparison with C. After 14 days of treatment, the relative

expression of this gene was increased in the FTH, TL and TH groups in comparison to the C group. In the F and FTL groups it was comparable with the C group.

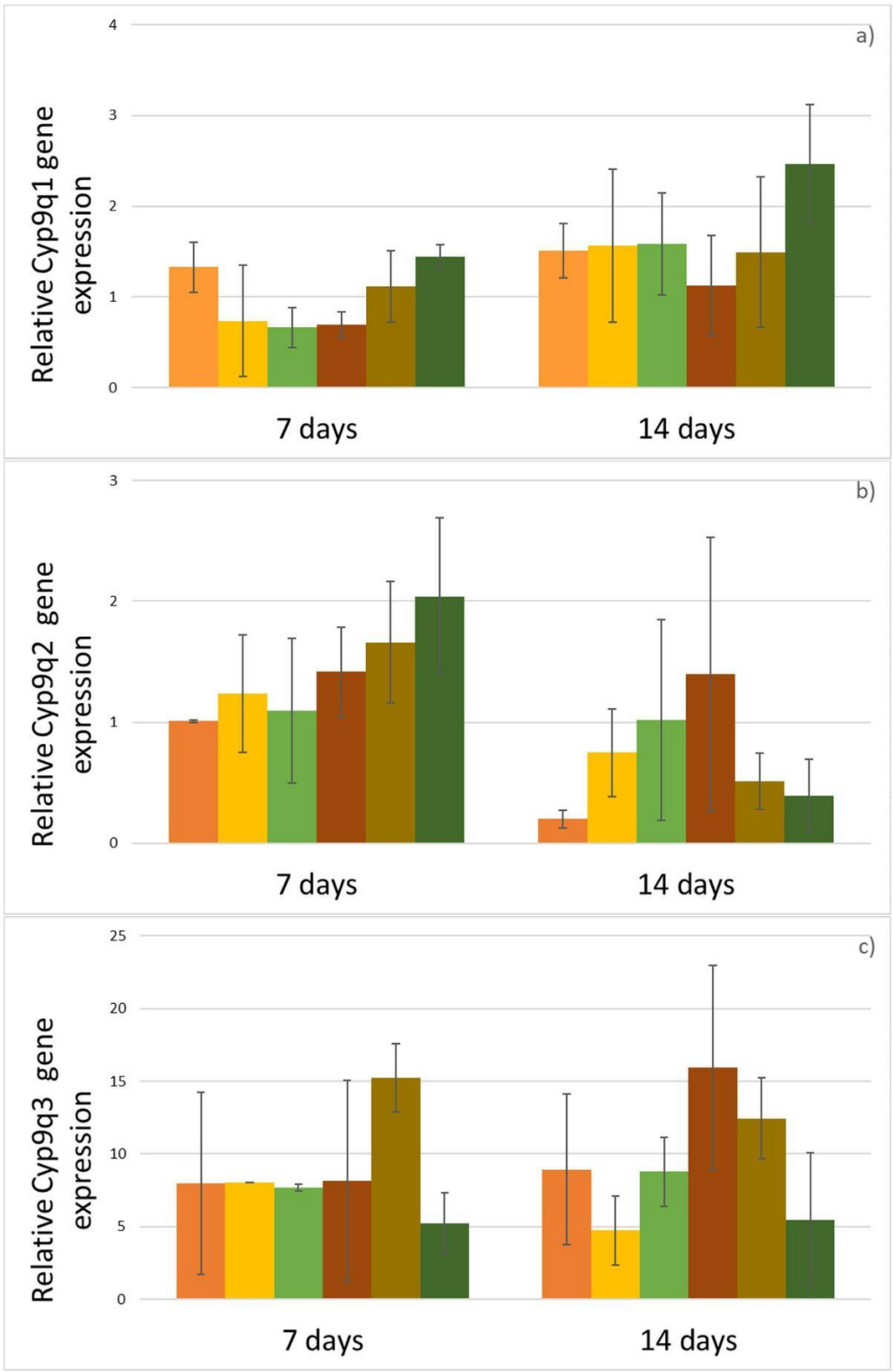


Figure 3. *Cont*.

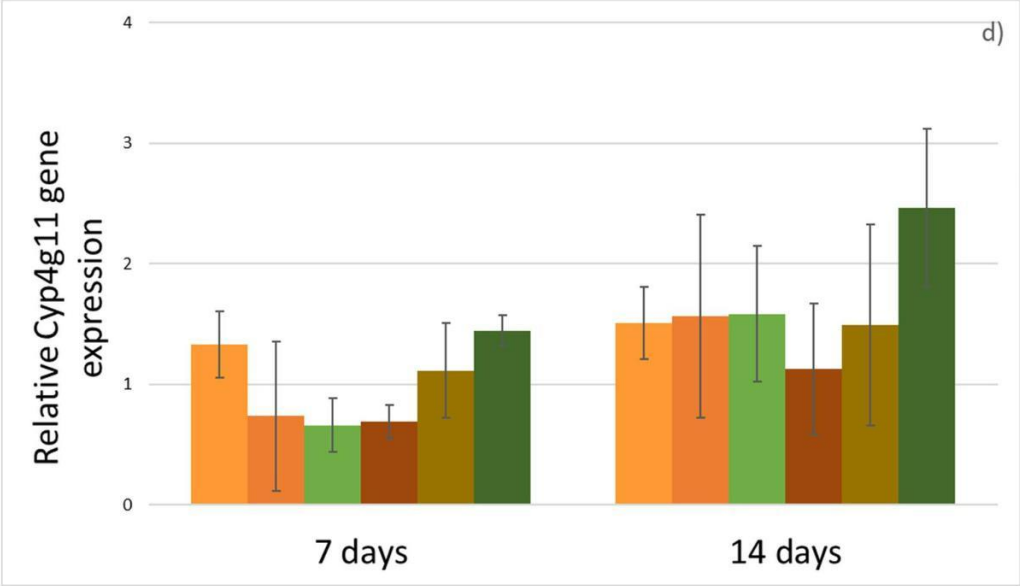


Figure 3. Relative expressions of four cytochrome P450 genes in bee workers depending on treatment at the 7th and 14th days of the experiment. Error bars denote two technical replications of three samples. (**a**) CYP9Q1 gene, (**b**)CYP9Q2 gene, (**c**) CYP9Q3 gene, (**d**) CYP4G11 genes.

4. Discussion

As expected, significantly higher mortality was observed in the treatment groups containing thiaclopride (TL, TH) compared to thiacloprid-free groups (C, F). This is consistent with the findings reported by Retschnig et al. [19]. However, Retschnig et al. [19] observed significantly lower mortality levels in bees intoxicated with high doses of thiacloprid than those observed in this study (TH). This difference may be due to different levels of sensitivity between bee subtaxons [20]. On the other hand, a low level of bee mortality was observed in group F, which did not differ from group C, indicating the safety of phenolic compounds for bees, which is in accordance with the results of Liao et al. [31]. Conversely, a statistically significant decrease was observed in the mortality rate of the group FTL in comparison with group TL, as well as in FTH compared to TH, which was probably caused by the increased detoxification capacity [30] and antioxidant activity [33] of the experimental bees due to phenolic-enriched diets [31]. The fact that with the addition of phenolic compounds (FTL), the mortality of intoxicated bees decreased significantly compared to the TL group, but did not reach the same level as in non-intoxicated bees (C), indicates the limited detoxification capacity in honey bees [28,42]. This trend was even more pronounced in the groups with a high dose of thiacloprid. A very significant reduction in mortality was observed with the addition of phenolic compounds (FTH) in comparison with the group without phenolic compounds (TH), but losses were still higher than in all other groups. The relationship between the experimental groups FTL, F and C seems to be an interesting phenomenon. No statistically significant difference in mortality was observed between the FTL and F groups, but there was a difference between the FTL and C groups. This can be explained by the probable increase of metabolic load caused by increased flavonoid levels [43].

The food consumption in the group containing phenolic compounds (F) was higher than in the group fed only with sucrose solution (C). Similar results were obtained by Porrini et al. [44]. They observed increased food intake when feeding bees with the addition of essential oils that contained phenolic compounds as their main components. Nevertheless, in their study, the rate of food consumption was lower in the control group, as well as in the experimental groups, compared to our study. This difference was probably caused by the difference in the carbohydrate concentration of the feed solution. A higher concentration of sugars in the feed leads to lower feed consumption, and vice versa [45].

On the other hand, in groups C and F, the food consumption rate was significantly higher than the groups with the addition of pesticide (TL and TH). This is consistent with the findings of Gregorc et al. [46] and Tosi et al. [47]. This was probably caused by increased levels of stress as a result of the addition of pesticides in the food [45]. However, in the case of intoxicated groups fed food enriched with phenolic compounds, the rate of food intake was significantly increased compared with groups without phenolic compounds, both in the case of low amounts of pesticide (FTL) and the case of high amounts of pesticide (FTH). This trend may be explained by the increased detoxification capacity [30] and antioxidant activity [33] caused by phenolic compounds in the food [31]. Differences in food consumption between TL and TH were not observed, nor were they observed between FTL and FTH. Therefore, the amount of pesticide in the food did not affect the level of consumption, which is consistent with the results of Retschnig et al. [19].

The relative expression of CYP9Q1, CYP9Q2, CYP9Q3 and CYP4G11 genes was analyzed using RT-qPCR. The cytochrome P450 enzyme group was chosen as the main endpoint in the detoxification process because it is responsible for the activity of the detoxification pathways of neonicotinoids [17]. The gene expression of four genes responsible for detoxication was analyzed after 7 and 14 days of treatment. In previous studies [30,37,48], bees were fed once with pesticide at the beginning of the experiment, and then the mortality and gene expression were analyzed in the first days after treatment. Conversely, in this study, a long-term experiment with long-term exposition to the tested substances was performed, with bees being fed continuously throughout the whole experiment. The expression levels of detoxification genes are highly dependent on time after pesticide treatment [49]. Therefore, the time of collection of genetic material could be the main reason why differences in detoxification gene expressions between experimental groups were not conclusive, and that the expression levels did not differ significantly between groups. Our results suggest a trend in which the expression in the F, FTL and C groups was comparable, and the gene expression in other intoxicated groups was increased. This could indicate that increased expression probably took place at the beginning of experiment, and that in the first days of bee sampling, the level of enzymes cytochrome P450 were already increased. However, better explanation of this issue could be provided by quantification of expressed protein. It would be suitable to carry out this investigation in future experiments.

Wheeler and Robinson [50] point out the problem that beekeepers use artificial bee food for bees, which, however, usually does not contain certain ingredients with high nutritional value and importance that are natural components of honey and pollen. Thus, it is clear that in addition to macronutrients (carbohydrates and proteins), the bee diet should also contain other elements (such as phenolic compounds) that have a conclusive impact on their detoxification capacity [30,31]. Based on the results of this study, we suggest that the addition of phenolic compounds to bee nutrition could to some extent increase the detoxification capacity of bees [30,31], which is often reduced due to malnutrition caused by degradation of the environment and the associated loss and contamination of food resources, as well as factors related to routine beekeeping management [4,50]. In addition, according to Mao et al. [29], some phenolic substances have an effect on the suppression of ovarian development, suggesting that phenolic substances could be used in the future to solve other problems in beekeeping practice.

5. Conclusions

Phenolic compounds, as natural components of the bee diet, have been demonstrated to have a positive impact on the longevity of honey bees intoxicated by thiacloprid, as well as their food intake.

The results of the experiments suggest that by adding phenolic substances to bee nutrition, the risks associated with the intoxication of bees can be reduced.

The expression levels of detoxification genes alone, depending on the treatment, may not be sufficient, and it is appropriate to support this with quantification of expressed proteins.

Author Contributions: Conceptualization, M.H.; Data curation, J.Š. and I.H.; Formal analysis, P.M., J.Š. and T.K.; Funding acquisition, V.Č. and A.B.; Investigation, P.M. and A.B.; Methodology, M.H. and I.H.; Project administration, A.B. and T.K.; Supervision, V.Č.; Validation, J.Š.; Writing—original draft, M.H., P.M., I.H. and T.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Mendel University in Brno, grant number AF–IGA2019–IP007 and Ministry of agriculture, grant number QK1910356.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The study did not report any data.

Acknowledgments: We thank Antonín Pr˘idal (Department of Zoology, Fisheries, Hydrobiology and Apiculture, Mendel University in Brno) for providing us with the used chemicals, the background of the university apiary, and apicultural consultations.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Aizen, M.A.; Garibaldi, L.A.; Cunningham, S.A.; Klein, A.M. How much does agriculture depend on pollinators? Lessons from long-term trends in crop production. *Ann. Bot.* **2009**, *103*, 1579–1588. [CrossRef]
2. Stokstad, E. The Case of the Empty Hives. *Science* **2007**, *316*, 970–972. [CrossRef]
3. Brodschneider, R.; Crailsheim, K. Nutrition and Health in Honey Bees. *Apidologie* **2010**, *41*, 278–294. [CrossRef]
4. Di Pasquale, G.; Salignon, M.; Le Conte, Y.; Belzunces, L.P.; Decourtye, A.; Kretzschmar, A.; Suchail, S.; Brunet, J.-L.; Alaux, C. Influence of Pollen Nutrition on Honey Bee Health: Do Pollen Quality and Diversity Matter? *PLoS ONE* **2013**, *8*, e72016. [CrossRef] [PubMed]
5. Sharpe, R.J.; Heyden, L.C. Honey Bee Colony Collapse Disorder Is Possibly Caused by a Dietary Pyrethrum Deficiency. *Biosci. Hypotheses* **2009**, *2*, 439–440. [CrossRef]
6. Haydak, M.H. Honey Bee Nutrition. *Annu. Rev. Entomol.* **1970**, *15*, 143–156. [CrossRef]
7. Naug, D. Nutritional Stress Due to Habitat Loss May Explain Recent Honeybee Colony Collapses. *Biol. Conserv.* **2009**, *142*, 2369–2372. [CrossRef]
8. Johnson, R.M.; Ellis, M.D.; Mullin, C.A.; Frazier, M. Pesticides and Honey Bee Toxicity—USA. *Apidologie* **2010**, *41*, 312–331. [CrossRef]
9. Decourtye, A.; Mader, E.; Desneux, N. Landscape Enhancement of Floral Resources for Honey Bees in Agro-Ecosystems. *Apidologie* **2010**, *41*, 264–277. [CrossRef]
10. Manning, R.; Rutkay, A.; Eaton, L.; Dell, B. Lipid-enhanced Pollen and Lipid-reduced Flour Diets and Their Effect on the Longevity of Honey Bees (*Apis mellifera* L.). *Aust. J. Entomol.* **2007**, *46*, 251–257. [CrossRef]
11. Hayes, J., Jr.; Underwood, R.M.; Pettis, J. A Survey of Honey Bee Colony Losses in the US, Fall 2007 to Spring 2008. *PLoS ONE* **2008**, *3*, e4071.
12. Wahl, O.; Ulm, K. Influence of Pollen Feeding and Physiological Condition on Pesticide Sensitivity of the Honey Bee *Apis mellifera* Carnica. *Oecologia* **1983**, *59*, 106–128. [CrossRef]
13. Alaux, C.; Ducloz, F.; Crauser, D.; Le Conte, Y. Diet Effects on Honeybee Immunocompetence. *Biol. Lett.* **2010**, *6*, 562–565. [CrossRef] [PubMed]
14. Keller, I.; Fluri, P.; Imdorf, A. Pollen Nutrition and Colony Development in Honey Bees—Part II. *Bee World* **2005**, *86*, 27–34. [CrossRef]
15. Frazier, M.; Mullin, C.; Frazier, J.; Ashcraft, S. What Have Pesticides Got to Do with It? *Am. Bee J.* **2008**, *148*, 521–524.
16. Medrzycki, P.; Sgolastra, F.; Bortolotti, L.; Bogo, G.; Tosi, S.; Padovani, E.; Porrini, C.; Sabatini, A.G. Influence of Brood Rearing Temperature on Honey Bee Development and Susceptibility to Poisoning by Pesticides. *J. Apic. Res.* **2010**, *49*, 52–59. [CrossRef]
17. Iwasa, T.; Motoyama, N.; Ambrose, J.T.; Roe, R.M. Mechanism for the Differential Toxicity of Neonicotinoid Insecticides in the Honey Bee, *Apis mellifera*. *Crop Prot.* **2004**, *23*, 371–378. [CrossRef]
18. Alaux, C.; Brunet, J.; Dussaubat, C.; Mondet, F.; Tchamitchan, S.; Cousin, M.; Brillard, J.; Baldy, A.; Belzunces, L.P.; Le Conte, Y. Interactions between Nosema Microspores and a Neonicotinoid Weaken Honeybees (*Apis mellifera*). *Environ. Microbiol.* **2010**, *12*, 774–782. [CrossRef]
19. Retschnig, G.; Neumann, P.; Williams, G.R. Thiacloprid–Nosema Ceranae Interactions in Honey Bees: Host Survivorship but Not Parasite Reproduction Is Dependent on Pesticide Dose. *J. Invertebr. Pathol.* **2014**, *118*, 18–19. [CrossRef]
20. Suchail, S.; Guez, D.; Belzunces, L.P. Characteristics of Imidacloprid Toxicity in Two *Apis mellifera* Subspecies. *Environ. Toxicol. Chem.* **2000**, *19*, 1901–1905. [CrossRef]
21. Nauen, R.; Ebbinghaus-Kintscher, U.; Schmuck, R. Toxicity and Nicotinic Acetylcholine Receptor Interaction of Imidacloprid and Its Metabolites in *Apis mellifera* (Hymenoptera: Apidae). *Pest Manag. Sci.* **2001**, *57*, 577–586. [CrossRef]

22. Wehling, M.; Ohe, W.; Brasse, D.; Forster, R. Colony Losses-Interactions of Plant Protection Products and Other Factors. *Jul. Kühn Arch.* **2009**, *423*, 153–154.
23. Grillone, G.; Laurino, D.; Manino, A.; Porporato, M. Toxicity of Thiametoxam on in vitro Reared Honey Bee Brood. *Apidologie* **2017**, *48*, 635–643. [CrossRef]
24. Osterman, J.; Wintermantel, D.; Locke, B.; Jonsson, O.; Semberg, E.; Onorati, P.; Forsgren, E.; Rosenkranz, P.; Rahbek-Pedersen, T.; Bommarco, R. Clothianidin Seed-Treatment Has No Detectable Negative Impact on Honeybee Colonies and Their Pathogens. *Nat. Commun.* **2019**, *10*, 1–13. [CrossRef] [PubMed]
25. Rundlöf, M.; Lundin, O. Can Costs of Pesticide Exposure for Bumblebees Be Balanced by Benefits from a Mass-Flowering Crop? *Environ. Sci. Technol.* **2019**, *53*, 14144–14151. [CrossRef]
26. Ghazoul, J. Buzziness as Usual? Questioning the Global Pollination Crisis. *Trends Ecol. Evol.* **2005**, *20*, 367–373. [CrossRef] [PubMed]
27. Alburaki, M.; Boutin, S.; Mercier, P.-L.; Loublier, Y.; Chagnon, M.; Derome, N. Neonicotinoid-Coated Zea Mays Seeds Indirectly Affect Honeybee Performance and Pathogen Susceptibility in Field Trials. *PLoS ONE* **2015**, *10*, e0125790. [CrossRef]
28. Claudianos, C.; Ranson, H.; Johnson, R.; Biswas, S.; Schuler, M.; Berenbaum, M.; Feyereisen, R.; Oakeshott, J.G. A Deficit of Detoxification Enzymes: Pesticide Sensitivity and Environmental Response in the Honeybee. *Insect Mol. Biol.* **2006**, *15*, 615–636. [CrossRef]
29. Mao, W.; Schuler, M.; Berenbaum, M. Task-related Differential Expression of Four Cytochrome P450 Genes in Honeybee Appendages. *Insect Mol. Biol.* **2015**, *24*, 582–588. [CrossRef] [PubMed]
30. Mao, W.; Schuler, M.A.; Berenbaum, M.R. Honey Constituents Up-Regulate Detoxification and Immunity Genes in the Western Honey Bee *Apis mellifera*. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 8842–8846. [CrossRef]
31. Liao, L.-H.; Wu, W.-Y.; Berenbaum, M.R. Impacts of Dietary Phytochemicals in the Presence and Absence of Pesticides on Longevity of Honey Bees (*Apis mellifera*). *Insects* **2017**, *8*, 22. [CrossRef]
32. Cianciosi, D.; Forbes-Hernández, T.Y.; Afrin, S.; Gasparrini, M.; Reboredo-Rodriguez, P.; Manna, P.P.; Zhang, J.; Bravo Lamas, L.; Martínez Flórez, S.; Agudo Toyos, P. Phenolic Compounds in Honey and Their Associated Health Benefits: A Review. *Molecules* **2018**, *23*, 2322. [CrossRef]
33. Moniruzzaman, M.; Yung An, C.; Rao, P.V.; Hawlader, M.N.I.; Azlan, S.A.B.M.; Sulaiman, S.A.; Gan, S.H. Identification of Phenolic Acids and Flavonoids in Monofloral Honey from Bangladesh by High Performance Liquid Chromatography: Determination of Antioxidant Capacity. *BioMed Res. Int.* **2014**, *2014*, 737490. [CrossRef]
34. Williams, G.R.; Alaux, C.; Costa, C.; Csaki, T.; Doublet, V.; Eisenhardt, D.; Brodschneider, R. Standard Methods for Maintaining Adult *Apis mellifera* in Cages under in Vitro Laboratory Conditions. *J. Apic. Res.* **2013**, *52*, 1–36. [CrossRef]
35. Lourenço, A.P.; Mackert, A.; dos Santos Cristino, A.; Simões, Z.L.P. Validation of Reference Genes for Gene Expression Studies in the Honey Bee, *Apis mellifera*, by Quantitative Real-Time RT-PCR. *Apidologie* **2008**, *39*, 372–385. [CrossRef]
36. Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2-ΔΔCT Method. *Methods* **2001**, *25*, 402–408. [CrossRef] [PubMed]
37. Mao, W.; Schuler, M.A.; Berenbaum, M.R. CYP9Q-Mediated Detoxification of Acaricides in the Honey Bee (*Apis mellifera*). *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 12657–12662. [CrossRef] [PubMed]
38. Calla, B.; MacLean, M.; Liao, L.; Dhanjal, I.; Tittiger, C.; Blomquist, G.J.; Berenbaum, M.R. Functional Characterization of CYP4G11—a Highly Conserved Enzyme in the Western Honey Bee *Apis mellifera*. *Insect Mol. Biol.* **2018**, *27*, 661–674. [CrossRef]
39. Tesovnik, T.; Cizelj, I.; Zorc, M.; Čitar, M.; Božič, J.; Glavan, G.; Narat, M. Immune Related Gene Expression in Worker Honey Bee (*Apis mellifera* Carnica) Pupae Exposed to Neonicotinoid Thiamethoxam and Varroa Mites (Varroa Destructor). *PLoS ONE* **2017**, *12*, e0187079. [CrossRef] [PubMed]
40. Kaplan, E.L.; Meier, P. Nonparametric Estimation from Incomplete Observations. *J. Am. Stat. Assoc.* **1958**, *53*, 457–481. [CrossRef]
41. Therneau, T.M.; Grambsch, P.M. The cox model. In *Modeling Survival Data: Extending the Cox Model*; Springer: New York, NY, USA, 2000; pp. 39–77.
42. Yu, S.J. Interactions of Allelochemicals with Detoxication Enzymes of Insecticide-Susceptible and Resistant Fall Armyworms. *Pestic. Biochem. Physiol.* **1984**, *22*, 60–68. [CrossRef]
43. Mao, W.; Schuler, M.A.; Berenbaum, M.R. Disruption of Quercetin Metabolism by Fungicide Affects Energy Production in Honey Bees (*Apis mellifera*). *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 2538–2543. [CrossRef] [PubMed]
44. Porrini, M.P.; Fernández, N.J.; Garrido, P.M.; Gende, L.B.; Medici, S.K.; Eguaras, M.J. In Vivo Evaluation of Antiparasitic Activity of Plant Extracts on Nosema Ceranae (Microsporidia). *Apidologie* **2011**, *42*, 700–707. [CrossRef]
45. Tong, L.; Nieh, J.C.; Tosi, S. Combined Nutritional Stress and a New Systemic Pesticide (Flupyradifurone, Sivanto®) Reduce Bee Survival, Food Consumption, Flight Success, and Thermoregulation. *Chemosphere* **2019**, *237*, 124408. [CrossRef]
46. Gregorc, A.; Alburaki, M.; Rinderer, N.; Sampson, B.; Knight, P.R.; Karim, S.; Adamczyk, J. Effects of Coumaphos and Imidacloprid on Honey Bee (Hymenoptera: Apidae) Lifespan and Antioxidant Gene Regulations in Laboratory Experiments. *Sci. Rep.* **2018**, *8*, 15003. [CrossRef]
47. Tosi, S.; Nieh, J.C.; Sgolastra, F.; Cabbri, R.; Medrzycki, P. Neonicotinoid Pesticides and Nutritional Stress Synergistically Reduce Survival in Honey Bees. *Proc. R. Soc. B* **2017**, *284*, 20171711. [CrossRef] [PubMed]
48. Manjon, C.; Troczka, B.J.; Zaworra, M.; Beadle, K.; Randall, E.; Hertlein, G.; Kumar, S.S.; Nauen, R. Unravelling the Molecular Determinants of Bee Sensitivity to Neonicotinoid Insecticides. *Curr. Biol.* **2018**, *28*, 1137–1143. [CrossRef]

49. Alptekin, S.; Bass, C.; Nicholls, C.; Paine, M.J.; Clark, S.J.; Field, L.; Moores, G.D. Induced Thiacloprid Insensitivity in Honeybees (*Apis mellifera* L.) Is Assoc. with Up-regulation of Detoxification Genes. *Insect Mol. Biol.* **2016**, *25*, 171–180. [CrossRef]
50. Wheeler, M.M.; Robinson, G.E. Diet-Dependent Gene Expression in Honey Bees: Honey vs. Sucrose or High Fructose Corn Syrup. *Sci. Rep.* **2014**, *4*, 5726. [CrossRef] [PubMed]

insects

MDPI

Article

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

Petr Mráz [1,*], Marian Hýbl [1], Marek Kopecký [1], Andrea Bohatá [1], Irena Hoštičková [1], Jan Šipoš [2], Kateřina Vočadlová [1] and Vladislav Čurn [1]

[1] Faculty of Agriculture, University of South Bohemia in Ceske Budejovice, Studentska 1668, 370 05 Ceske Budejovice, Czech Republic; mario.eko@seznam.cz (M.H.); mkopecky@zf.jcu.cz (M.K.); bohata@zf.jcu.cz (A.B.); jelini00@zf.jcu.cz (I.H.); katerina.vocadlova@gmail.com (K.V.); curn@zf.jcu.cz (V.Č.)

[2] Faculty of Agronomy, Mendel University in Brno, Zemedelska 1, 613 00 Brno, Czech Republic; jan.sipos@mendelu.cz

* Correspondence: mrazpe01@zf.jcu.cz

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.

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

Citation: Mráz, P.; Hýbl, M.; Kopecký, M.; Bohatá, A.; Hoštičková, I.; Šipoš, J.; Vočadlová, K.; Čurn, V. Screening of Honey Bee Pathogens in the Czech Republic and Their Prevalence in Various Habitats. *Insects* **2021**, *12*, 1051. https://doi.org/10.3390/insects12121051

Academic Editors: Ivana Tlak Gajger, Franco Mutinelli and Cristina Botías

Received: 1 November 2021
Accepted: 23 November 2021
Published: 24 November 2021

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 Melliflcae	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

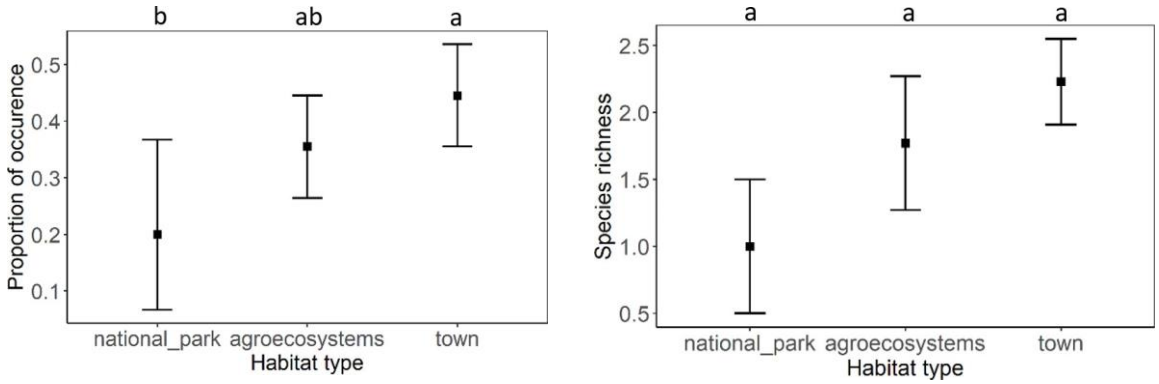
To evaluate whether pathogen occurrence and species richness differ among honey bee colonies and habitat types, we used separate generalized liner mixed-effects models (GLMM) [59]. In the case when species richness was used as dependent variable, GLMM with a Gaussian error distribution was used. When 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).

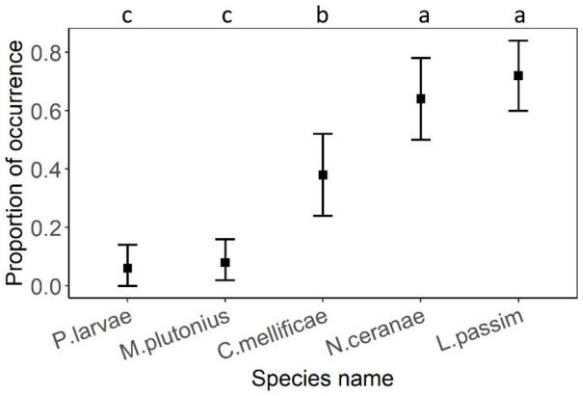


Figure 2. The comparison of proportion of eukaryotic pathogen occurrence regardless of habitat. Black squares represent means and the error bars represent 95% confidence intervals. Significant differences (<0.05) are indicated by different letters.

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).

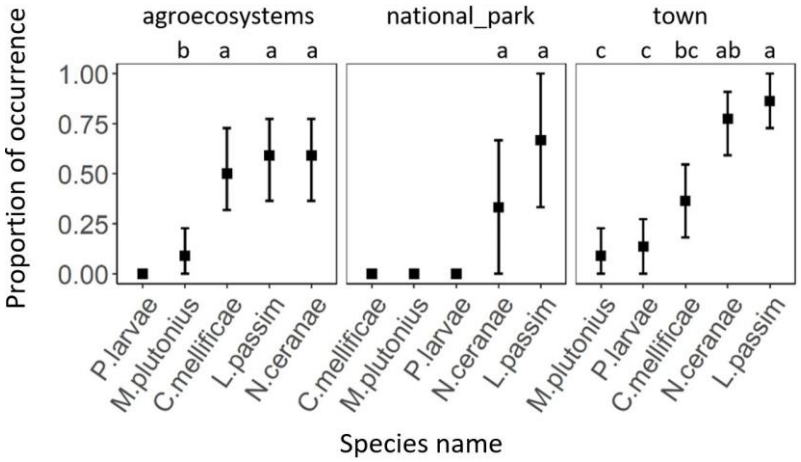
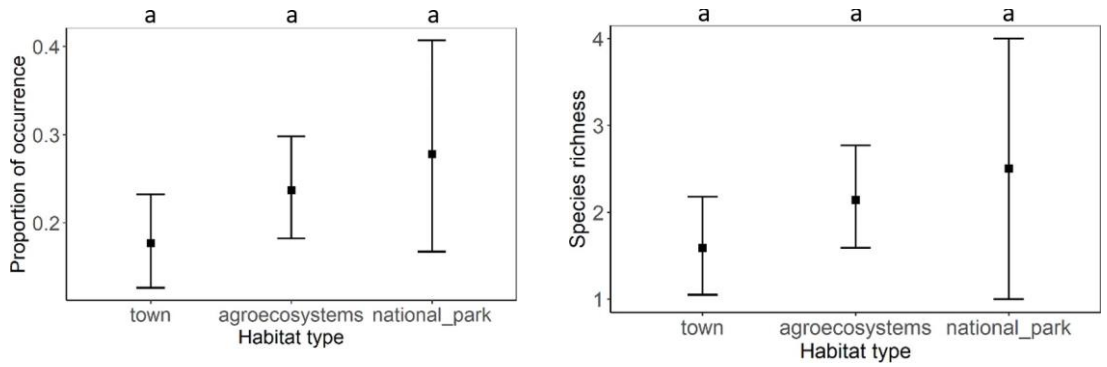


Figure 3. The comparison of proportion of eukaryotic pathogen occurrence within each habitat type. Black squares represent means and the error bars represent 95% confidence intervals. Significant differences (<0.05) are indicated by different letters.

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.

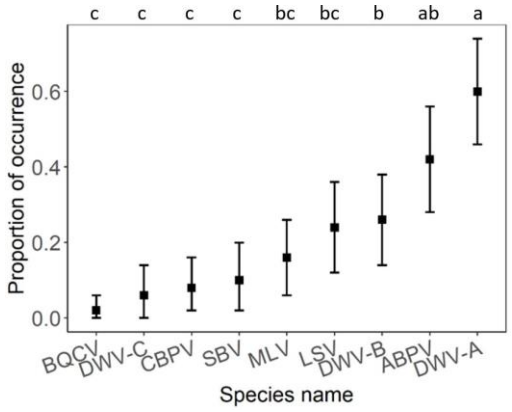


Figure 5. The comparison of proportion of viral pathogen occurrence regardless of habitat. Black squares represent means and the error bars represent 95% confidence intervals. Significant differences (<0.05) are indicated by different letters.

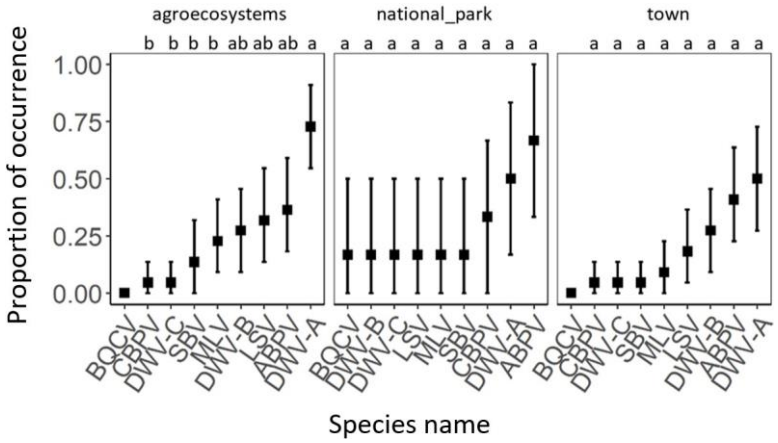


Figure 6. The comparison of proportion of viral pathogen occurrence within each habitat type. Black squares represent means and the error bars represent 95% confidence intervals. Significant differences (<0.05) are indicated by different letters.

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%).

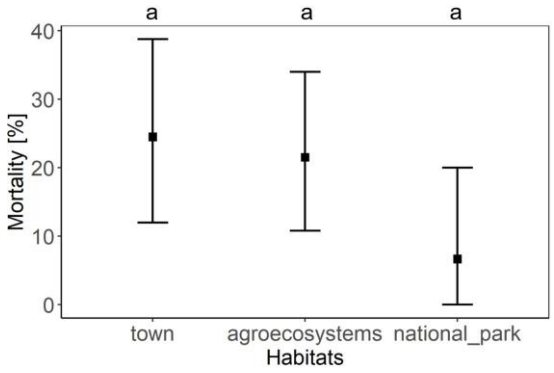


Figure 7. The comparison of honey bee winter mortality rate according to type of habitat. Black squares represent means and the error bars represent 95% confidence intervals.

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).

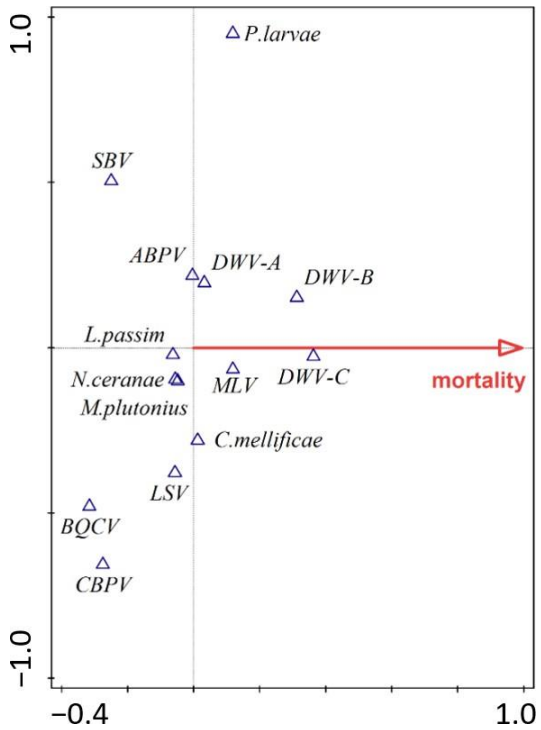


Figure 8. Partial canonical correspondence analysis biplot with the habitat type as a covariable showing the strength of the association of individual pathogens with the mortality rate.

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.

Author Contributions: Conceptualization, P.M. and M.H.; Data curation, M.K.; K.V., I.H. and J.Š.; Formal analysis, A.B., V.Č. and J.Š.; Investigation, P.M., M.K. and I.H.; Methodology, P.M. and K.V.; Supervision, A.B. and V.Č.; Writing—original draft, P.M. and M.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Ministry of Agriculture, grant number QK1910356, and University of South Bohemia, grant number GAJU027/2019/Z.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The study did not report any data.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Gallai, N.; Salles, J.-M.; Settele, J.; Vaissière, B.E. Economic Valuation of the Vulnerability of World Agriculture Confronted withPollinator Decline. *Ecol. Econ.* **2009**, *68*, 810–821. [CrossRef]
2. Stokstad, E. The Case of the Empty Hives. *Science* **2007**, *316*, 970–972. [CrossRef]
3. Cox-Foster, D.L.; Conlan, S.; Holmes, E.C.; Palacios, G.; Evans, J.D.; Moran, N.A.; Quan, P.-L.; Briese, T.; Hornig, M.; Geiser, D.M. A Metagenomic Survey of Microbes in Honey Bee Colony Collapse Disorder. *Science* **2007**, *318*, 283–287. [CrossRef]
4. Higes, M.; Martín-Hernández, R.; Garrido-Bailón, E.; González-Porto, A.V.; García-Palencia, P.; Meana, A.; Del Nozal, M.J.; Mayo, R.; Bernal, J.L. Honeybee Colony Collapse Due to *Nosema ceranae* in Professional Apiaries. *Environ. Microbiol. Rep.* **2009**, *1*, 110–113. [CrossRef]
5. Alonso-Salces, R.M.; Cugnata, N.M.; Guaspari, E.; Pellegrini, M.C.; Aubone, I.; De Piano, F.G.; Antunez, K.; Fuselli, S.R. Natural Strategies for the Control of *Paenibacillus larvae*, the Causative Agent of American Foulbrood in Honey Bees: A Review. *Apidologie* **2017**, *48*, 387–400. [CrossRef]
6. Frazier, M.; Mullin, C.; Frazier, J.; Ashcraft, S. What Have Pesticides Got to Do with It? *Am. Bee J.* **2008**, *148*, 521–524.
7. Mutinelli, F.; Granato, A. La Sindrome Del Collasso Della Colonia (Colony Collapse Disorder) Negli USA. *Aggiorn. Sulla Situaz. Attuale. Apoidea* **2007**, *4*, 175–185.
8. Sharpe, R.J.; Heyden, L.C. Honey Bee Colony Collapse Disorder Is Possibly Caused by a Dietary Pyrethrum Deficiency. *Biosci. Hypotheses* **2009**, *2*, 439–440. [CrossRef]
9. Alaux, C.; Ducloz, F.; Crauser, D.; Le Conte, Y. Diet Effects on Honeybee Immunocompetence. *Biol. Lett.* **2010**, *6*, 562–565. [CrossRef]
10. Genersch, E.; Von Der Ohe, W.; Kaatz, H.; Schroeder, A.; Otten, C.; Büchler, R.; Berg, S.; Ritter, W.; Mühlen, W.; Gisder, S. The German Bee Monitoring Project: A Long Term Study to Understand Periodically High Winter Losses of Honey Bee Colonies. *Apidologie* **2010**, *41*, 332–352. [CrossRef]
11. Lanzi, G.; De Miranda, J.R.; Boniotti, M.B.; Cameron, C.E.; Lavazza, A.; Capucci, L.; Camazine, S.M.; Rossi, C. Molecular and Biological Characterization of Deformed Wing Virus of Honeybees (*Apis mellifera* L.). *J. Virol.* **2006**, *80*, 4998–5009. [CrossRef][PubMed]
12. Mordecai, G.J.; Wilfert, L.; Martin, S.J.; Jones, I.M.; Schroeder, D.C. Diversity in a Honey Bee Pathogen: First Report of a Third Master Variant of the Deformed Wing Virus Quasispecies. *ISME J.* **2016**, *10*, 1264–1273. [CrossRef] [PubMed]
13. Dainat, B.; Evans, J.D.; Chen, Y.P.; Gauthier, L.; Neumann, P. Dead or Alive: Deformed Wing Virus and *Varroa destructor* Reduce the Life Span of Winter Honeybees. *Appl. Environ. Microbiol.* **2012**, *78*, 981–987. [CrossRef]
14. Martin, S.J.; Highfield, A.C.; Brettell, L.; Villalobos, E.M.; Budge, G.E.; Powell, M.; Nikaido, S.; Schroeder, D.C. Global Honey Bee Viral Landscape Altered by a Parasitic Mite. *Science* **2012**, *336*, 1304–1306. [CrossRef] [PubMed]
15. Schroeder, D.C.; Martin, S.J. Deformed Wing Virus: The Main Suspect in Unexplained Honeybee Deaths Worldwide. *Virulence* **2012**, *3*, 589–591. [CrossRef]
16. Mordecai, G.J.; Brettell, L.E.; Martin, S.J.; Dixon, D.; Jones, I.M.; Schroeder, D.C. Superinfection Exclusion and the Long-Term Survival of Honey Bees in Varroa-Infested Colonies. *ISME J.* **2016**, *10*, 1182–1191. [CrossRef]
17. Natsopoulou, M.E.; McMahon, D.P.; Doublet, V.; Frey, E.; Rosenkranz, P.; Paxton, R.J. The Virulent, Emerging Genotype B of Deformed Wing Virus Is Closely Linked to Overwinter Honeybee Worker Loss. *Sci. Rep.* **2017**, *7*, 5242. [CrossRef]
18. Ongus, J.R.; Peters, D.; Bonmatin, J.-M.; Bengsch, E.; Vlak, J.M.; van Oers, M.M. Complete Sequence of a Picorna-like Virus of the Genus Iflavirus Replicating in the Mite *Varroa destructor*. *J. Gen. Virol.* **2004**, *85*, 3747–3755. [CrossRef]
19. McMahon, D.P.; Fürst, M.A.; Caspar, J.; Theodorou, P.; Brown, M.J.; Paxton, R.J. A Sting in the Spit: Widespread Cross-infection of Multiple RNA Viruses across Wild and Managed Bees. *J. Anim. Ecol.* **2015**, *84*, 615–624. [CrossRef]
20. Tehel, A.; Brown, M.J.; Paxton, R.J. Impact of Managed Honey Bee Viruses on Wild Bees. *Curr. Opin. Virol.* **2016**, *19*, 16–22. [CrossRef]
21. Beims, H.; Bunk, B.; Erler, S.; Mohr, K.I.; Spröer, C.; Pradella, S.; Günther, G.; Rohde, M.; von der Ohe, W.; Steinert, M. Discovery of *Paenibacillus larvae* ERIC V: Phenotypic and Genomic Comparison to Genotypes ERIC I-IV Reveal Different Inventories of Virulence Factors Which Correlate with Epidemiological Prevalences of American Foulbrood. *Int. J. Med Microbiol.* **2020**, *310*, 151394. [CrossRef]
22. Genersch, E. American Foulbrood in Honeybees and Its Causative Agent, *Paenibacillus larvae*. *J. Invertebr. Pathol.* **2010**, *103*, S10–S19. [CrossRef] [PubMed]
23. Reybroeck, W. Residues of Antibiotics and Chemotherapeutics in Honey. *J. Apic. Res.* **2018**, *57*, 97–112. [CrossRef]
24. Forsgren, E. European Foulbrood in Honey Bees. *J. Invertebr. Pathol.* **2010**, *103*, S5–S9. [CrossRef] [PubMed]
25. Lewkowski, O.; Erler, S. Virulence of *Melissococcus plutonius* and Secondary Invaders Associated with European Foulbrood Disease of the Honey Bee. *MicrobiologyOpen* **2019**, *8*, e00649. [CrossRef] [PubMed]
26. Erban, T.; Ledvinka, O.; Kamler, M.; Hortova, B.; Nesvorna, M.; Tyl, J.; Titera, D.; Markovic, M.; Hubert, J. Bacterial Community Associated with Worker Honeybees (*Apis mellifera*) Affected by European Foulbrood. *PeerJ* **2017**, *5*, e3816. [CrossRef] [PubMed]

27. Martín-Hernández, R.; Bartolomé, C.; Chejanovsky, N.; Le Conte, Y.; Dalmon, A.; Dussaubat, C.; García-Palencia, P.; Meana, A.; Pinto, M.A.; Soroker, V. *Nosema ceranae* in *Apis mellifera*: A 12 Years Postdetection Perspective. *Environ. Microbiol.* **2018**, *20*, 1302–1329. [CrossRef]
28. Spiltoir, C.F. Life Cycle of *Ascosphaera apis* (Pericystis Apis). *Am. J. Bot.* **1955**, 501–508. [CrossRef]
29. Mráz, P.; Hýbl, M.; Kopecký, M.; Bohatá, A.; Konopická, J.; Hoštičková, I.; Konvalina, P.; Šipoš, J.; Rost, M.; Čurn, V. The Effect of Artificial Media and Temperature on the Growth and Development of the Honey Bee Brood Pathogen *Ascosphaera apis*. *Biology* **2021**, *10*, 431. [CrossRef]
30. Chantawannakul, P.; Puchanichanthranon, T.; Wongsiri, S. Inhibitory Effects of Some Medicinal Plant Extracts on the Growth of *Ascosphaera apis*. In Proceedings of the III WOCMAP Congress on Medicinal and Aromatic Plants-Volume 4: Targeted Screening of Medicinal and Aromatic Plants, Economics 678, Chiang Mai, Thailand, 3–7 February 2003; pp. 183–189.
31. Ansari, M.J.; Al-Ghamdi, A.; Usmani, S.; Khan, K.A.; Alqarni, A.S.; Kaur, M.; Al-Waili, N. In Vitro Evaluation of the Effects of Some Plant Essential Oils on *Ascosphaera apis*, the Causative Agent of Chalkbrood Disease. *Saudi J. Biol. Sci.* **2017**, *24*, 1001–1006. [CrossRef]
32. Aronstein, K.A.; Murray, K.D. Chalkbrood Disease in Honey Bees. *J. Invertebr. Pathol.* **2010**, *103*, S20–S29. [CrossRef]
33. Schwarz, R.S.; Bauchan, G.R.; Murphy, C.A.; Ravoet, J.; de Graaf, D.C.; Evans, J.D. Characterization of Two Species of Trypanosomatidae from the Honey Bee *Apis mellifera*: *Crithidia mellificae* Langridge and McGhee, and *Lotmaria passim* n. Gen., n. Sp. *J. Eukaryot. Microbiol.* **2015**, *62*, 567–583. [CrossRef]
34. Langridge, D.; McGhee, R.B. *Crithidia mellificae* n. Sp. an Acidophilic Trypanosomatid of the Honey Bee *Apis mellifera*. *J. Protozool.* **1967**, *14*, 485–487. [CrossRef]
35. Vejnovic, B.; Stevanovic, J.; Schwarz, R.S.; Aleksic, N.; Mirilovic, M.; Jovanovic, N.M.; Stanimirovic, Z. Quantitative PCR Assessment of *Lotmaria passim* in *Apis mellifera* Colonies Co-Infected Naturally with *Nosema ceranae*. *J. Invertebr. Pathol.* **2018**, *151*, 76–81. [CrossRef]
36. Ravoet, J.; Maharramov, J.; Meeus, I.; De Smet, L.; Wenseleers, T.; Smagghe, G.; De Graaf, D.C. Comprehensive Bee Pathogen Screening in Belgium Reveals *Crithidia mellificae* as a New Contributory Factor to Winter Mortality. *PLoS ONE* **2013**, *8*, e72443.
37. Arismendi, N.; Caro, S.; Castro, M.P.; Vargas, M.; Riveros, G.; Venegas, T. Impact of Mixed Infections of Gut Parasites *Lotmaria passim* and *Nosema ceranae* on the Lifespan and Immune-Related Biomarkers in *Apis mellifera*. *Insects* **2020**, *11*, 420. [CrossRef] [PubMed]
38. Castelli, L.; Branchiccela, B.; Invernizzi, C.; Tomasco, I.; Basualdo, M.; Rodriguez, M.; Zunino, P.; Antúnez, K. Detection of *Lotmaria passim* in Africanized and European Honey Bees from Uruguay, Argentina and Chile. *J. Invertebr. Pathol.* **2019**, *160*, 95–97. [CrossRef]
39. Raymann, K.; Moran, N.A. The Role of the Gut Microbiome in Health and Disease of Adult Honey Bee Workers. *Curr. Opin. Insect Sci.* **2018**, *26*, 97–104. [CrossRef]
40. Hubert, J.; Bicianova, M.; Ledvinka, O.; Kamler, M.; Lester, P.J.; Nesvorna, M.; Kopecky, J.; Erban, T. Changes in the Bacteriome of Honey Bees Associated with the Parasite *Varroa destructor*, and Pathogens *Nosema* and *Lotmaria passim*. *Microb. Ecol.* **2017**, *73*, 685–698. [CrossRef]
41. Hristov, P.; Shumkova, R.; Palova, N.; Neov, B. Factors Associated with Honey Bee Colony Losses: A Mini-Review. *Vet. Sci.* **2020**, *7*, 166. [CrossRef] [PubMed]
42. Hýbl, M.; Mráz, P.; Šipoš, J.; Hoštičková, I.; Bohatá, A.; Čurn, V.; Kopec, T. Polyphenols as Food Supplement Improved Food Consumption and Longevity of Honey Bees (*Apis mellifera*) Intoxicated by Pesticide Thiacloprid. *Insects* **2021**, *12*, 572. [CrossRef]
43. Brodschneider, R.; Crailsheim, K. Nutrition and Health in Honey Bees. *Apidologie* **2010**, *41*, 278–294. [CrossRef]
44. Rutter, L.; Carrillo-Tripp, J.; Bonning, B.C.; Cook, D.; Toth, A.L.; Dolezal, A.G. Transcriptomic Responses to Diet Quality and Viral Infection in *Apis mellifera*. *BMC Genom.* **2019**, *20*, 412. [CrossRef] [PubMed]
45. Naug, D. Nutritional Stress Due to Habitat Loss May Explain Recent Honeybee Colony Collapses. *Biol. Conserv.* **2009**, *142*, 2369–2372. [CrossRef]
46. Garbuzov, M.; Schürch, R.; Ratnieks, F.L. Eating Locally: Dance Decoding Demonstrates That Urban Honey Bees in Brighton, UK, Forage Mainly in the Surrounding Urban Area. *Urban Ecosyst.* **2015**, *18*, 411–418. [CrossRef]
47. Hybl, M.; Mraz, P.; Sipos, J. Diversity of Bees (Apoidea) and Their Pesticide Contamination in Two Different Types of Agricultural Management. In Proceedings of the MendelNet, Brno, Czech Republic, 11–12 November 2020; Volume 27, pp. 216–221.
48. Ryba, S.; Titera, D.; Schodelbauerova-Traxmandlova, I.; Kindlmann, P. Prevalence of Honeybee Viruses in the Czech Republic and Coinfections with Other Honeybee Disease. *Biologia* **2012**, *67*, 590–595. [CrossRef]
49. Bakonyi, T.; Derakhshifar, I.; Grabensteiner, E.; Nowotny, N. Development and Evaluation of PCR Assays for the Detection of *Paenibacillus larvae* in Honey Samples: Comparison with Isolation and Biochemical Characterization. *Appl. Environ. Microbiol.* **2003**, *69*, 1504–1510. [CrossRef]
50. Govan, V.; Allsopp, M.; Davison, S. A PCR Detection Method for Rapid Identification of *Paenibacillus larvae*. *Appl. Environ. Microbiol.* **1999**, *65*, 2243–2245. [CrossRef]
51. Garrido-Bailón, E.; Higes, M.; Martínez-Salvador, A.; Antúnez, K.; Botías, C.; Meana, A.; Prieto, L.; Martín-Hernández, R. The Prevalence of the Honeybee Brood Pathogens A *Scosphaera apis*, P *Aenibacillus larvae* and M *Elissococcus plutonius* in S Panish Apiaries Determined with a New Multiplex PCR Assay. *Microb. Biotechnol.* **2013**, *6*, 731–739. [CrossRef]

52. Stevanovic, J.; Schwarz, R.S.; Vejnovic, B.; Evans, J.D.; Irwin, R.E.; Glavinic, U.; Stanimirovic, Z. Species-Specific Diagnostics of *Apis mellifera* Trypanosomatids: A Nine-Year Survey (2007–2015) for Trypanosomatids and Microsporidians in Serbian Honey Bees. *J. Invertebr. Pathol.* **2016**, *139*, 6–11. [CrossRef]
53. Arismendi, N.; Bruna, A.; Zapata, N.; Vargas, M. PCR-Specific Detection of Recently Described *Lotmaria passim* (Trypanosomatidae) in Chilean Apiaries. *J. Invertebr. Pathol.* **2016**, *134*, 1–5. [CrossRef]
54. Riveros, G.; Arismendi, N.; Zapata, N.; Evans, D.; Pérez, I.; Aldea, P.; Vargas, M. Occurrence, Prevalence and Viral Load of Deformed Wing Virus Variants in *Apis mellifera* Colonies in Chile. *J. Apic. Res.* **2020**, *59*, 63–68. [CrossRef]
55. Sguazza, G.H.; Reynaldi, F.J.; Galosi, C.M.; Pecoraro, M.R. Simultaneous Detection of Bee Viruses by Multiplex PCR. *J. Virol. Methods* **2013**, *194*, 102–106. [CrossRef] [PubMed]
56. Tentcheva, D.; Gauthier, L.; Zappulla, N.; Dainat, B.; Cousserans, F.; Colin, M.E.; Bergoin, M. Prevalence and Seasonal Variations of Six Bee Viruses in *Apis mellifera* L. and *Varroa destructor* Mite Populations in France. *Appl. Environ. Microbiol.* **2004**, *70*, 7185–7191. [CrossRef]
57. Iwanowicz, D.D.; Wu-Smart, J.Y.; Olgun, T.; Smart, A.H.; Otto, C.R.; Lopez, D.; Evans, J.D.; Cornman, R. An Updated Genetic Marker for Detection of Lake Sinai Virus and Metagenetic Applications. *PeerJ* **2020**, *8*, e9424. [CrossRef] [PubMed]
58. Gauthier, L.; Ravallec, M.; Tournaire, M.; Cousserans, F.; Bergoin, M.; Dainat, B.; de Miranda, J.R. Viruses Associated with Ovarian Degeneration in *Apis mellifera* L. Queens. *PLoS ONE* **2011**, *6*, e16217. [CrossRef]
59. Bates, D.; Mächler, M.; Bolker, B.; Walker, S. Fitting Linear Mixed-Effects Models Using Lme4. *arXiv* **2014**, arXiv:1406.5823.
60. ter Braak, C.J.; Smilauer, P. Canoco Reference Manual and User's Guide: Software for Ordination, Version 5.0. 2012. Available online: https://research.wur.nl/en/publications/canoco-reference-manual-and-users-guide-software-for-ordination-v (accessed on 22 September 2021).
61. Gómez-Moracho, T.; Buendía-Abad, M.; Benito, M.; García-Palencia, P.; Barrios, L.; Bartolomé, C.; Maside, X.; Meana, A.; Jiménez-Antón, M.D.; Olías-Molero, A.I. Experimental Evidence of Harmful Effects of *Crithidia mellificae* and *Lotmaria passim* on Honey Bees. *Int. J. Parasitol.* **2020**, *50*, 1117–1124. [CrossRef]
62. Michalczyk, M.; Bancerz-Kisiel, A.; Sokół, R. *Lotmaria passim* as Third Parasite Gastrointestinal Tract of Honey Bees Living in Tree Trunk. *J. Apic. Sci.* **2020**, *64*, 143–151. [CrossRef]
63. Cepero, A.; Ravoet, J.; Gómez-Moracho, T.; Bernal, J.L.; Del Nozal, M.J.; Bartolomé, C.; Maside, X.; Meana, A.; González-Porto, A.V.; de Graaf, D.C. Holistic Screening of Collapsing Honey Bee Colonies in Spain: A Case Study. *BMC Res. Notes* **2014**, *7*, 649. [CrossRef]
64. Higes, M.; Martín-Hernández, R.; Botías, C.; Bailón, E.G.; González-Porto, A.V.; Barrios, L.; Del Nozal, M.J.; Bernal, J.L.; Jiménez, J.J.; Palencia, P.G. How Natural Infection by *Nosema ceranae* Causes Honeybee Colony Collapse. *Environ. Microbiol.* **2008**, *10*, 2659–2669. [CrossRef] [PubMed]
65. Gisder, S.; Hedtke, K.; Möckel, N.; Frielitz, M.-C.; Linde, A.; Genersch, E. Five-Year Cohort Study of *Nosema* Spp. in Germany: Does Climate Shape Virulence and Assertiveness of *Nosema ceranae*? *Appl. Environ. Microbiol.* **2010**, *76*, 3032–3038. [CrossRef] [PubMed]
66. Stevanovic, J.; Stanimirovic, Z.; Genersch, E.; Kovacevic, S.R.; Ljubenkovic, J.; Radakovic, M.; Aleksic, N. Dominance of *Nosema ceranae* in Honey Bees in the Balkan Countries in the Absence of Symptoms of Colony Collapse Disorder. *Apidologie* **2011**, *42*, 49–58. [CrossRef]
67. Higes, M.; Meana, A.; Bartolomé, C.; Botías, C.; Martín-Hernández, R. *Nosema ceranae* (Microsporidia), a Controversial 21st Century Honey Bee Pathogen. *Environ. Microbiol. Rep.* **2013**, *5*, 17–29. [CrossRef] [PubMed]
68. Paxton, R.J.; Klee, J.; Korpela, S.; Fries, I. *Nosema ceranae* Has Infected *Apis mellifera* in Europe since at Least 1998 and May Be More Virulent than *Nosema apis*. *Apidologie* **2007**, *38*, 558–565. [CrossRef]
69. Gisder, S.; Schüler, V.; Horchler, L.L.; Groth, D.; Genersch, E. Long-Term Temporal Trends of *Nosema* Spp. Infection Prevalence in Northeast Germany: Continuous Spread of *Nosema ceranae*, an Emerging Pathogen of Honey Bees (*Apis mellifera*), but No General Replacement of *Nosema apis*. *Front. Cell. Infect. Microbiol.* **2017**, *7*, 301. [CrossRef]
70. Shumkova, R.; Georgieva, A.; Radoslavov, G.; Sirakova, D.; Dzhebir, G.; Neov, B.; Bouga, M.; Hristov, P. The First Report of the Prevalence of *Nosema ceranae* in Bulgaria. *PeerJ* **2018**, *6*, e4252. [CrossRef]
71. Ostroverkhova, N.V.; Konusova, O.L.; Kucher, A.N.; Kireeva, T.N.; Rosseykina, S.A. Prevalence of the Microsporidian *Nosema* Spp. in Honey Bee Populations (*Apis mellifera*) in Some Ecological Regions of North Asia. *Vet. Sci.* **2020**, *7*, 111. [CrossRef]
72. Li, Z.; You, X.; Wang, L.; Yan, Z.; Zhou, Z. Spore Morphology and Ultrastructure of an *Ascosphaera apis* Strain from the Honeybees (*Apis mellifera*) in Southwest China. *Mycologia* **2018**, *110*, 325–338. [CrossRef]
73. Biová, J.; Bzdil, J.; Dostálková, S.; Petrivalsky, M.; Brus, J.; Carra, E.; Danihlík, J. American Foulbrood in the Czech Republic: ERIC II Genotype of *Paenibacillus larvae* Is Prevalent. *Front. Vet. Sci.* **2021**, *8*, 698976. [CrossRef]
74. Erban, T.; Ledvinka, O.; Kamler, M.; Hortova, B.; Nesvorna, M.; Tyl, J.; Titera, D.; Markovic, M.; Hubert, J. European Foulbrood in Czechia after 40 Years: Application of next-Generation Sequencing to Analyze *Melissococcus plutonius* Transmission and Influence on the Bacteriome of *Apis mellifera*. *PeerJ Prepr.* **2017**, *4*, e2618v1.
75. Budge, G.E.; Barrett, B.; Jones, B.; Pietravalle, S.; Marris, G.; Chantawannakul, P.; Thwaites, R.; Hall, J.; Cuthbertson, A.G.; Brown, M.A. The Occurrence of *Melissococcus plutonius* in Healthy Colonies of *Apis mellifera* and the Efficacy of European Foulbrood Control Measures. *J. Invertebr. Pathol.* **2010**, *105*, 164–170. [CrossRef] [PubMed]
76. Roy, C.; Franco, S. Investigation of an Atypical Case of European Foulbrood in France. *Vet. Rec. Case Rep.* **2021**, *9*, e45. [CrossRef]

77. Grangier, V.; Belloy, L.; Charrière, J.-D.; Doherr, M.G.; Fritsche, A.; Waldvogel, A.S. Real-Time PCR as a Decision Aid in the Control of European Foulbrood. *J. Apic. Res.* **2015**, *54*, 366–372. [CrossRef]
78. Kevill, J.L.; de Souza, F.S.; Sharples, C.; Oliver, R.; Schroeder, D.C.; Martin, S.J. DWV-A Lethal to Honey Bees (*Apis mellifera*): A Colony Level Survey of DWV Variants (A, B, and C) in England, Wales, and 32 States across the US. *Viruses* **2019**, *11*, 426. [CrossRef]
79. Olgun, T.; Everhart, S.E.; Anderson, T.; Wu-Smart, J. Comparative Analysis of Viruses in Four Bee Species Collected from Agricultural, Urban, and Natural Landscapes. *PLoS ONE* **2020**, *15*, e0234431.
80. McMahon, D.P.; Natsopoulou, M.E.; Doublet, V.; Fürst, M.; Weging, S.; Brown, M.J.; Gogol-Döring, A.; Paxton, R.J. Elevated Virulence of an Emerging Viral Genotype as a Driver of Honeybee Loss. *Proc. R. Soc. B Biol. Sci.* **2016**, *283*, 20160811. [CrossRef]
81. Tehel, A.; Vu, Q.; Bigot, D.; Gogol-Döring, A.; Koch, P.; Jenkins, C.; Doublet, V.; Theodorou, P.; Paxton, R. The Two Prevalent Genotypes of an Emerging Infectious Disease, Deformed Wing Virus, Cause Equally Low Pupal Mortality and Equally High Wing Deformities in Host Honey Bees. *Viruses* **2019**, *11*, 114. [CrossRef]
82. Gisder, S.; Möckel, N.; Eisenhardt, D.; Genersch, E. In Vivo Evolution of Viral Virulence: Switching of Deformed Wing Virus between Hosts Results in Virulence Changes and Sequence Shifts. *Environ. Microbiol.* **2018**, *20*, 4612–4628. [CrossRef]
83. Ryabov, E.V.; Childers, A.K.; Chen, Y.; Madella, S.; Nessa, A.; Evans, J.D. Recent Spread of *Varroa destructor* Virus-1, a Honey Bee Pathogen, in the United States. *Sci. Rep.* **2017**, *7*, 17447. [CrossRef]
84. Manley, R.; Temperton, B.; Doyle, T.; Gates, D.; Hedges, S.; Boots, M.; Wilfert, L. Knock-on Community Impacts of a Novel Vector: Spillover of Emerging DWV-B from Varroa-infested Honeybees to Wild Bumblebees. *Ecol. Lett.* **2019**, *22*, 1306–1315. [CrossRef]
85. Kevill, J.L.; Highfield, A.; Mordecai, G.J.; Martin, S.J.; Schroeder, D.C. ABC Assay: Method Development and Application to Quantify the Role of Three DWV Master Variants in Overwinter Colony Losses of European Honey Bees. *Viruses* **2017**, *9*, 314. [CrossRef]
86. Berthoud, H.; Imdorf, A.; Haueter, M.; Radloff, S.; Neumann, P. Virus Infections and Winter Losses of Honey Bee Colonies (*Apis mellifera*). *J. Apic. Res.* **2010**, *49*, 60–65. [CrossRef]
87. Nguyen, B.K.; Ribière, M.; vanEngelsdorp, D.; Snoeck, C.; Saegerman, C.; Kalkstein, A.L.; Schurr, F.; Brostaux, Y.; Faucon, J.-P.; Haubruge, E. Effects of Honey Bee Virus Prevalence, *Varroa destructor* Load and Queen Condition on Honey Bee Colony Survival over the Winter in Belgium. *J. Apic. Res.* **2011**, *50*, 195–202. [CrossRef]
88. Faurot-Daniels, C.; Glenny, W.; Daughenbaugh, K.F.; McMenamin, A.J.; Burkle, L.A.; Flenniken, M.L. Longitudinal Monitoring of Honey Bee Colonies Reveals Dynamic Nature of Virus Abundance and Indicates a Negative Impact of Lake Sinai Virus 2 on Colony Health. *PLoS ONE* **2020**, *15*, e0237544. [CrossRef]
89. De Miranda, J.R.; Cornman, R.S.; Evans, J.D.; Semberg, E.; Haddad, N.; Neumann, P.; Gauthier, L. Genome Characterization, Prevalence and Distribution of a Macula-like Virus from *Apis mellifera* and *Varroa destructor*. *Viruses* **2015**, *7*, 3586–3602. [CrossRef]
90. McMenamin, A.J.; Flenniken, M.L. Recently Identified Bee Viruses and Their Impact on Bee Pollinators. *Curr. Opin. Insect Sci.* **2018**, *26*, 120–129. [CrossRef]
91. Abou Kubaa, R.; Molinatto, G.; Khaled, B.; Daher-Hjaij, N.; Heinoun, K.; Saponari, M. First Detection of Black Queen Cell Virus, *Varroa destructor* Macula-like Virus, *Apis mellifera* Filamentous Virus and *Nosema ceranae* in Syrian Honey Bees *Apis mellifera* Syriaca. *Bull. Insectol.* **2018**, *71*, 217–224.
92. von Büren, R.S.; Oehen, B.; Kuhn, N.J.; Erler, S. High-Resolution Maps of Swiss Apiaries and Their Applicability to Study Spatial Distribution of Bacterial Honey Bee Brood Diseases. *PeerJ* **2019**, *7*, e6393. [CrossRef]
93. Brodschneider, R.; Brus, J.; Danihlík, J. Comparison of Apiculture and Winter Mortality of Honey Bee Colonies (*Apis mellifera*) in Austria and Czechia. *Agric. Ecosyst. Environ.* **2019**, *274*, 24–32. [CrossRef]
94. Taric, E.; Glavinic, U.; Vejnovic, B.; Stanojkovic, A.; Aleksic, N.; Dimitrijevic, V.; Stanimirovic, Z. Oxidative Stress, Endoparasite Prevalence and Social Immunity in Bee Colonies Kept Traditionally vs. Those Kept for Commercial Purposes. *Insects* **2020**, *11*, 266. [CrossRef] [PubMed]
95. Tehel, A.; Streicher, T.; Tragust, S.; Paxton, R.J. Experimental Infection of Bumblebees with Honeybee-Associated Viruses: No Direct Fitness Costs but Potential Future Threats to Novel Wild Bee Hosts. *R. Soc. Open Sci.* **2020**, *7*, 200480. [CrossRef] [PubMed]
96. Simone-Finstrom, M.; Li-Byarlay, H.; Huang, M.H.; Strand, M.K.; Rueppell, O.; Tarpy, D.R. Migratory Management and Environmental Conditions Affect Lifespan and Oxidative Stress in Honey Bees. *Sci. Rep.* **2016**, *6*, 32023. [CrossRef] [PubMed]
97. Graystock, P.; Ng, W.H.; Parks, K.; Tripodi, A.D.; Muñiz, P.A.; Fersch, A.A.; Myers, C.R.; McFrederick, Q.S.; McArt, S.H. Dominant Bee Species and Floral Abundance Drive Parasite Temporal Dynamics in Plant-Pollinator Communities. *Nat. Ecol. Evol.* **2020**, *4*, 1358–1367. [CrossRef]

biology

MDPI

Article

The Effect of Artificial Media and Temperature on the Growth and Development of the Honey Bee Brood Pathogen *Ascosphaera apis*

Petr Mráz [1,*], Marian Hýbl [2], Marek Kopecký [3], Andrea Bohatá [4], Jana Konopická [1,5], Irena Hoštičková [1], Petr Konvalina [3], Jan Šipoš [2], Michael Rost [1] and Vladislav Čurn [1]

[1] Department of Genetics and Agricultural Biotechnology, Faculty of Agriculture, University of South Bohemia in Ceske Budejovice, Studentska 1668, 370 05 Ceske Budejovice, Czech Republic; konopj01@zf.jcu.cz (J.K.); jelini00@zf.jcu.cz (I.H.); rost@zf.jcu.cz (M.R.); curn@zf.jcu.cz (V.Č.)
[2] Department of Zoology, Fisheries, Hydrobiology and Apiculture, Faculty of AgriSciences, Mendel University in Brno, Zemedelska 1, 613 00 Brno, Czech Republic; mario.eko@seznam.cz (M.H.); jan.sipos@mendelu.cz (J.Š.)
[3] Department of Agroecosystems, Faculty of Agriculture, University of South Bohemia in Ceske Budejovice, Studentska 1668, 370 05 Ceske Budejovice, Czech Republic; mkopecky@zf.jcu.cz (M.K.); konvalina@zf.jcu.cz (P.K.)
[4] Department of Crop Production, Faculty of Agriculture, University of South Bohemia in Ceske Budejovice, Studentska 1668, 370 05 Ceske Budejovice, Czech Republic; bohata@zf.jcu.cz
[5] Biology Centre of the Czech Academy of Sciences, Institute of Entomology, 370 05 Ceske Budejovice, Czech Republic
* Correspondence: mrazpe01@zf.jcu.cz

Citation: Mráz, P.; Hýbl, M.; Kopecký, M.; Bohatá, A.; Konopická, J.; Hoštičková, I.; Konvalina, P.; Šipoš, J.; Rost, M.; Čurn, V. The Effect of Artificial Media and Temperature on the Growth and Development of the Honey Bee Brood Pathogen *Ascosphaera apis*. *Biology* **2021**, *10*, 431. https://doi.org/10.3390/biology10050431

Academic Editor: Gill Diamond

Received: 5 April 2021
Accepted: 8 May 2021
Published: 12 May 2021

Simple Summary: Chalkbrood is a worldwide spread honey bee brood disease caused by the fungal pathogen *Ascosphaera apis*. The disease is commonly treated with fungicides, but due to the accumulation of residues, these fungicides have been banned in many countries, including European Union countries. Since then, control of chalkbrood has been problematic. The disease is fatal to individual honey bee larvae and can cause significant losses in terms of both bee numbers and colony productivity, and can even in some cases lead to colony collapse. Owing to these reasons, in vitro fungus cultivation is necessary to properly understand its pathogenesis as well as life cycle for the possible future development of an efficient and environmentally friendly control method. Therefore, in this study, several artificial media and different temperatures were evaluated to see their impact on the growth and development of *A. apis*. Furthermore, one of the media was modified by the addition of crushed honey bee brood to simulate natural conditions. This medium was found to be the most suitable for fungus reproductive structure production. In addition, a biological pattern was found explaining the relationships between temperature and the size of the fungal reproductive structures.

Abstract: *Ascosphaera apis* is a causative agent of chalkbrood, which is one of the most widespread honey bee diseases. In our experiments, the influence of several artificial media and cultivation under different temperatures was evaluated. Concretely, the radial growth of separated mating types was measured, reproductive structures in a Neubauer hemocytometer chamber were counted simultaneously, and the morphometry of spore cysts and spore balls was assessed. The complex set of experiments determined suitable cultivation conditions. A specific pattern between reproductive structure size and temperature was found. The optimal temperature for both mating types was 30 °C. SDA and YGPSA media are suitable for fast mycelial growth. Moreover, the effect of bee brood on fungus growth and development in vitro was investigated by modification of culture medium. The newly modified medium PDA-BB4 was most effective for the production of the reproductive structures. The result suggests that honey bee brood provides necessary nutrients for proper fungus development during in vitro cultivation. As there is no registered therapeutic agent against chalkbrood in most countries, including the European Union, the assessment of *A.*

apis growth and development in different conditions could help to understand fungus pathogenesis and thus control chalkbrood disease.

Keywords: *Apis mellifera*; chalkbrood; cultivation; culture media; sporulation

1. Introduction

The western honey bee (*Apis mellifera*) is one of the most important pollinators of many agricultural crops as well as herbaceous plants [1–3]. However, a sudden large decline in the number of honey bees has been observed worldwide [4,5]. Especially in the United States of America, the number of bee colonies has decreased by over 50% since the 1940s [2,6,7]. Many factors are responsible for reducing bee colonies' vitality and viability, including the application of acaricides, fungicides, and antibiotics [8], agrochemicals [9,10], malnutrition [11,12], inappropriate beekeeping practices or changes in habitat [5,13], and, especially, bee diseases [14,15]. One of the pathogens harming colonies is a widespread heterothallic fungus, *Ascosphaera apis* (Maasen ex Claussen) L.S. Olive and Spiltoir, which causes chalkbrood disease [16].

Ascosphaera apis (Ascomycota, Eurotiomycetes, Ascosphaerales) is a species closely adapted to honey bees and can only invade bee brood. It was widely accepted that *A. apis* spores cannot germinate on the larval cuticle, therefore, the infection of bee brood starts when the spores are ingested with the food by bee larvae during their feeding [14]. The mode of larval infection distinguishes this species from other entomopathogenic fungi belonging to the order Hypocreales, which are able to penetrate the insect cuticle without the need for ingestion [17,18]. For this reason, it is not possible to utilize many well-known methods that are used for studying common entomopathogenic fungi [19]. After ingestion, the spores of *A. apis* need to be exposed to higher CO_2 concentrations to start germination [20]. In this case, CO_2 is produced by the larval tissues in the gut. The optimal temperature for spore germination is 35 °C [21]. After their activation, spores become swollen and create germ tubes that grow into dichotomous hyphae. Mycelium then penetrates through the peritrophic membrane of bee larvae into the body cavity and grows to the posterior end, where it breaks the barrier. In the case of the presence of both mating types, it starts to create spore cysts (ascomata). Ascospores, which are the only infective units causing chalkbrood [22], are formed in spore balls (asci) and located in resistant cysts [16]. The spores contain two nuclei; the bigger one lies in the center and the second smaller one is situated near the end of the spore [23]. The three-layered spore wall is tough, containing chitin as its major component [23,24], which helps ascospores stay viable for many years [20,25,26].

The first clinical signs of the disease are dead larvae that are covered by a fluffy white mold and they are usually swollen. After some time, they shrink and turn black/gray or white, depending on the presence of reproductive structures [26–28]. At the end of the fungus development cycle, honey bee larvae become mummified. Chalkbrood can be easily recognized by visual detection of these mummified bee brood, known as chalkbrood mummies, on the bottom board of beehives, as well as in uncapped cells. However, De Jong [29] claims that a low infestation level of this disease (less than 12% infection) is not recognizable, because worker bees remove the infected bee brood.

Chalkbrood is considered to be a stress-related disease [27,30,31], which could be the reason for its increased occurrence in recent years [22,23], because honey bees are stressed by many factors. According to Vojvodic et al. [31], chalkbrood is a chronic disease because it persists in beehives for a long time and can break out at any time, depending on the conditions, which makes this disease more dangerous. Chalkbrood is also considered to be more prevalent in damp weather conditions [32–34], fluctuating temperatures [35], or if the bee colonies are excessively fed with sugar syrup [36]. Brood chilling also causes stress and leads to outbreaks of the disease [21,31,37,38]. This situa-

tion can occur during rapid colony growth due to a lot of bee brood in combs and a relatively small number of worker bees taking care of the brood and warming the space [29,38] up to a temperature close to the optimal bee brood temperature of 35 °C [27].

There are some indications that this fungus, which is one of the most contagious and destructive bee brood pathogens [14], may be increasing in occurrence [22]. There are also some locations, such as northern Thailand [39] and China [23], where this pathogen causes high bee mortality because the even less pathogen-susceptible *Apis cerana cerana* can be easily infected by *A. apis* [40]. This fact contributes to chalkbrood's worldwide occurrence and adds to the importance of the disease. Although chalkbrood is fatal to individual bee larvae, it rarely causes colony losses [22]. However, in most cases, it causes significant losses of bee numbers and colony productivity [30,41], which makes it an economically important disease [27].

There is no registered therapeutic agent against chalkbrood disease [42] and it is difficult to involve or find any effective control agent [26,43] despite the promising results with plant essential oils recently [44,45]. Thus, for these reasons, it is necessary to understand the life cycle and pathogenesis of *A. apis*. From this point of view, the optimization of cultivation methods and the establishment of a stable and effective in vitro cultivation system with a high yield of ascospores are crucial.

Fungi have evolved several specialized strategies for overcoming insect defense mechanisms and reaching nutrients, including biotrophy (nutrition derived only from living cells, which ceases once the cell has died), necrotrophy (killing and utilization of dead tissues), and hemibiotrophy (initially biotrophic and then becoming necrotrophic). When they invade an appropriate kind of insect, the host provides them with the whole spectrum of nutrients they need [46]. Artificial media significantly influence the germination, growth, enzyme production, and thus also the virulence of entomopathogenic fungi [47,48]. Nutrition for fungi cultivated in vitro depends especially on the C/N ratio. Yeast extract and peptone have C/N ratios of 3.6:1 and 8:1, respectively, and represent different carbon and nitrogen sources [49]. According to Ibrahim et al.,2002 [47], nutrient-rich media such as SDA and YEA promote greater germination compared to nutrient-poor media. However, it can vary significantly depending on the species [49,50].

In the present study, commonly used artificial media as well as different temperatures were tested for the pathogen cultivation, with the aim of determining the optimal conditions for *A. apis* and verifying their influence on the reproductive structures' yield and morphometry. Moreover, the effect of bee brood addition on fungus growth and sporulation was compared. These findings are necessary to understand fungus pathogenesis and occurrence in beehives and the biological mechanisms of reducing spore loads in nature.

2. Materials and Methods

2.1. Fungal Isolate

Dry mummified bee brood (Figure 1) were collected from an apiary in the South Bohemian Region, Czech Republic, from the bottom boards of infected colonies and stored in a refrigerator (at 5 °C) until use. The mummies were crushed and small pieces were placed on potato dextrose agar (PDA) for spore activation. The plates were incubated at 35 °C until the growth of mycelia and subsequent sporulation were observed. During that time, ascospores of this fungus were activated and the opposite mating types observed. Male (−) and female (+) mating types were separated by continuous subculturing (Figure 2) until pure cultures were obtained. The opposite mating types were verified by a dual test (Figure 3), when reproductive structures (Figure 4) in lines between mycelia of opposite mating types were developed.

Figure 1. Mummified honey bee brood.

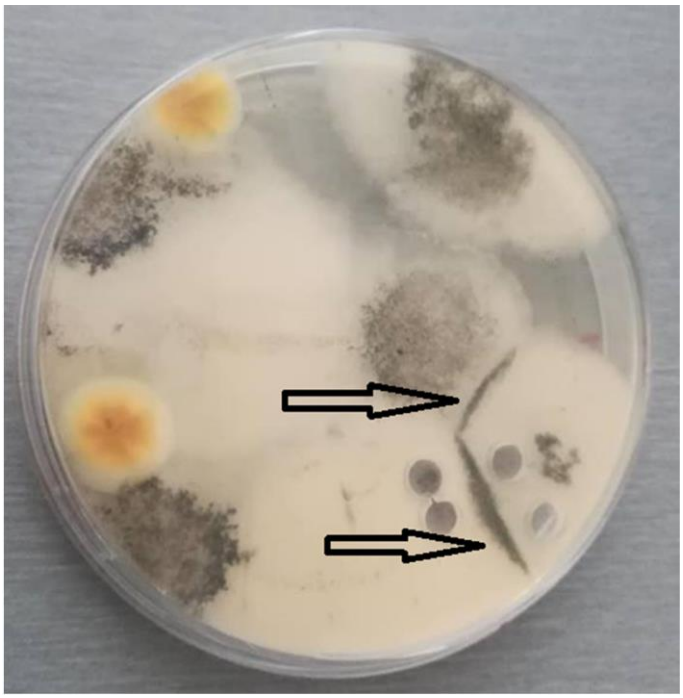


Figure 2. Separation of opposite mating types of *A. apis* by subcultivation. The mummified honey bee brood were crushed and subcultivated on PDA medium until black lines of reproductive structures appeared (shown by the two arrows). These lines indicated the presence of opposite mating types of *A. apis*. Mycelium was taken by a cork borer from that area.

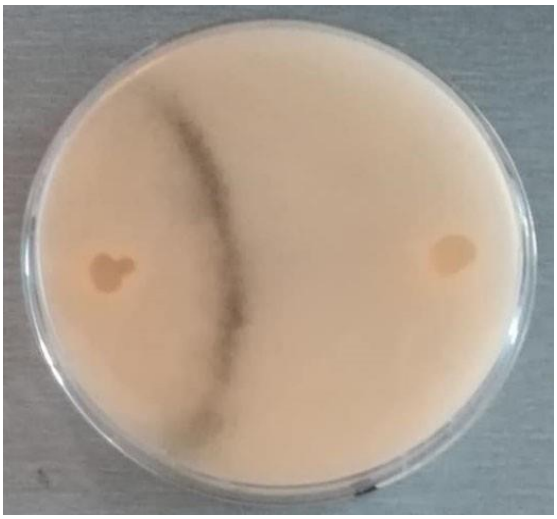


Figure 3. Pure cultures of opposite mating types of *A. apis* in dual test. Mycelia of opposite mating types were subjected to dual tests several times until pure cultures were established. The black line of reproductive structures is closer to the left side because of the slower growing (+) mating type.

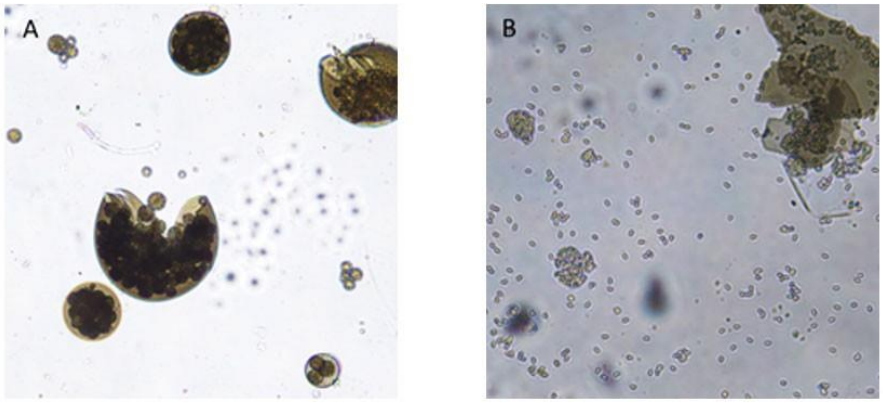


Figure 4. Spore cysts (**A**), spore balls (**A**), and ascospores (**B**). Reproductive structures of *A. apis* consist of spore cysts in which ascospores are formed in spore balls. If the cysts mature, they will open and ascospores can be released.

Maternal cultures of both mating types were maintained separately on PDA at 30 °C. The fungus was identified by molecular techniques. PCR and sequencing of the ITS1-5.8S-ITS2 (ITS) region were carried out. Genomic DNA of *A. apis* was extracted and used as a template for PCR identification. The final volume was 20 μL, including 1 μL of template DNA, 10 μL PPP Master Mix (Top-Bio, Vestec, Czech Republic), 7 μL of PCR Ultra H_2O (Top-Bio), and the specific ITS primers AscosFOR (TGTGTCTGTGCGGCTAGGTG) and AscosREV (GCTAGCCAGGGGGGAACTAA), 1 μL each at 10 μM [51]. PCR was performed under the following conditions: initial template denaturation at 95 °C for 2 min; 35 cycles at 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 45 s, with a final 7 min extension at 72 °C. PCR products were electrophoretically separated on 1.5% agarose gel, visualized by a UV transilluminator (INGENIUS, Trigon-plus, SYNGENE, Cestlice, Czech Republic), purified using a gel extraction kit following the manufacturer's instructions, and sent to SEQme (Dobris, Czech Republic) for data sequencing. Species identity was analyzed in the BLAST search tool available through the website of the NCBI [52] and confirmed by phylogenetic analysis. The ITS dataset used in this study was obtained from GenBank [53]. The *Aspergillus terreus* ITS

sequence (accession number NR_149331) was used as an outgroup for the analysis. Sequence data were aligned using the CLUSTALw algorithm as implemented in Geneious 8.1.9. MrBayes was run for 100,000 generations with 50,000 sample points under default prior probability settings.

Seven standard artificial media, namely potato dextrose agar, (PDA), Sabouraud dextrose agar (SDA), malt extract agar (MEA), Czapek dox agar (CDA), potato dextrose agar with 0.4% yeast extract (PDAY), and yeast glucose starch agar (YGPSA) (all from HiMedia Laboratories Pvt. Ltd., Prague, Czech Republic), and Iso-Sensitest agar (Oxoid ISA) (Thermo Scientific™, Pardubice, Czech Republic) were used. Three modified media were prepared by the addition of crushed frozen drone bee brood to PDA before sterilization (PDA-BB4: 40 g/L PDA; PDA-BB8: 80 g/L PDA; PDA-BB12: 120 g/L PDA). All the media were autoclaved at 121 °C for 45 min. Precipitates of crushed bee brood were removed by sterile cotton before pouring to plates.

2.2. Growth Parameters

Seven-day-old cultures of each mating type were used in the experiments. The disks were cut from the edge of growing mycelium using an 8 mm diameter cork borer. One disk of the mating type (+) or mating type (−) was placed in the middle of each tested medium in Petri dishes (90 mm). All plates were placed into plastic bags to keep a stable humidity (>90%) and avoid water evaporation from the media. All the variants were incubated at different temperatures (25 °C, 30 °C, and 35 °C) for six days. Four replications of each variant were performed. Colony diameter was measured daily with a 1 mm precision rule by two perpendicular lines across the bottom of each Petri plate crossing over the inoculum plug until the mycelia reached the edge of plate (84 mm in diameter) or ceased growth (Figure 5).

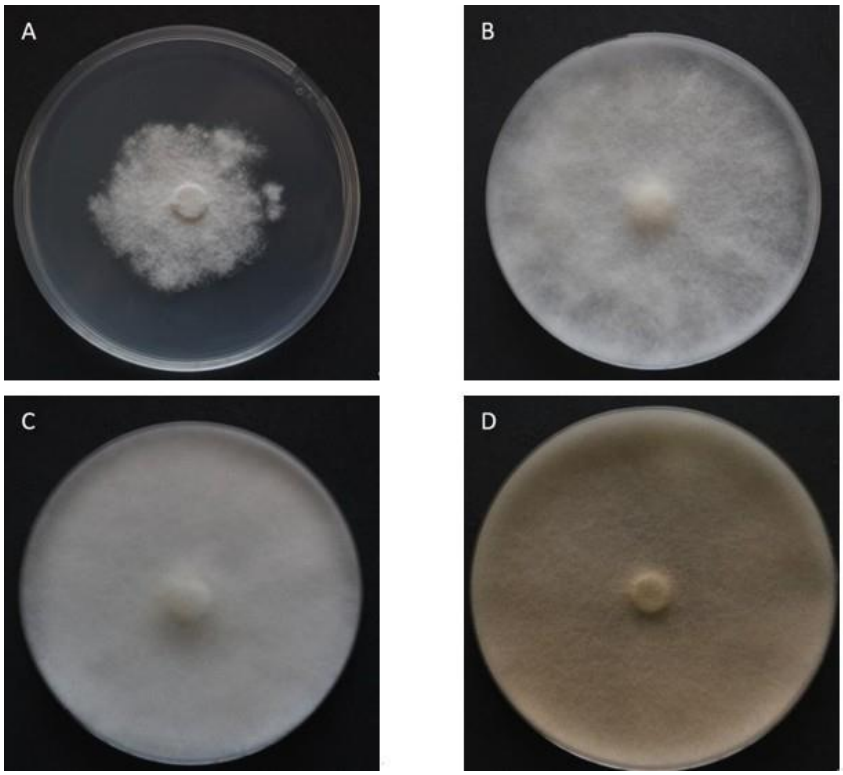


Figure 5. Growth differences among cultures of *A. apis* in different conditions. (**A**) Pure culture of (+) mating type of *A. apis* 15 days post inoculation on PDA at 35 °C (slow and irregular radial growth). (**B**) Pure culture of (+) mating type of *A.*

apis 15 days post inoculation on PDA at 25 °C (moderate radial growth). (**C**) Pure culture of (+) mating type of *A. apis* 15 days post inoculation on PDA at 30 °C (fast radial growth). (**D**) Pure culture of (−) mating type of *A. apis* 15 days post inoculation on PDA at 30 °C (fastest radial growth and quick aging).

2.3. Reproductive Structure Production

For the production of reproductive structures, six media (PDA, PDAY, SDA, MEA, YGPSA, and PDA-BB4) were selected based on the results of the previous experiment. One disk of mating type (+) and one disk of mating type (−) were placed symmetrically 5 cm apart from each other on all tested media. Four replications were prepared for each variant. All the variants were incubated at 25 °C, 30 °C, and 35 °C in plastic bags to keep a stable humidity (>90%). After 15 days of incubation, two disks (Ø 8 mm) were cut from the fully sporulated area at distances of 20 and 40 mm from the edge of each plate (a total of eight samples from each variant). Disks were separately transferred into a 1.5 mL microcentrifugation tube with 0.5 mL of 0.05% sterile water with Tween 80. The reproductive structures were washed out using a vortex for 60 s. The concentrations of cysts, spore balls, and ascospores from the obtained suspension were counted simultaneously in a Neubauer hemocytometer chamber (Sigma-Aldrich, Prague, Czech Republic) under a light microscope (Olympus CH20, Prague, Czech Republic) at 100× magnification (spore cysts and spore balls) and at 200× magnification (ascospores). The concentration of reproductive structures was recalculated for 1 mm^2 of a fully sporulated area.

2.4. Morphometry of Reproductive Structures

The same suspensions prepared for counting of reproductive structures also were used for morphometry of spore cysts and spore ball assessment. An aliquot of 50 µL of the suspension was deposited on the surface of the glass slide and the reproductive structures were observed at 200× magnification under a light microscope (Nikon Eclipse E200, Prague, Czech Republic). Altogether, 15 cysts and 15 spore balls for each sample (120 cysts and 120 spore balls per variant) were measured from the captured images (Nikon, Prague, Czech Republic) by two perpendicular diameters with the software NIS Elements E200. The range of sizes was determined and the mean size for both structures was calculated for each variant.

2.5. Statistical Analysis

Three experimental designs with repeated measures on the Petri dish were used for exploring how *A. apis* grows and develops in response to several factors: (a) a 3 × 10 × 2 factorial design was applied to test the effect of temperature and medium on radial growth of two mating types on the fourth day; (b) a 3 × 6 × 3 factorial design was applied to test the effect of temperature and medium on the production of the reproductive structures (spore cysts, spore balls, and spores), and (c) a 3 × 6 × 2 factorial design was applied to test the effect of temperature and medium on the size of the reproductive structures (spore cysts and spore balls). Mating types, temperature, medium, reproductive structures, and their interaction were entered into the models as fixed effects, and Petri dishes were entered as random effects. For data analysis, repeated-measures nonparametric ANOVA was used, which is a robust statistical tool for the analysis of multiple factorial designs with non-normal residuals. Before using ANOVA itself, the data were transformed by the "art" function (ARTool package) [54]. This function first aligns the data for each effect (main or interaction) and then assigns averaged ranks [55]. The post hoc comparison of the main effect for food source was conducted by the "emmeans" package with Bonferroni-corrected *p*-values [56].

The radial colony growth was analyzed by the generalized estimating equation (GEE) approach with Poisson error distribution and log link function [57]. The repeated measures from the same Petri dish were partitioned within clusters by the argument "id" in the GEE method. Temporal autocorrelation between repeated measures was adjusted

by specifying a first-order autocorrelation error covariance matrix. All analyses were performed using the statistical software R version 4.0.1 (R Core Team, 2020).

3. Results

We isolated two fungal strains from mummified honey bee larvae which were morphologically determined as *A. apis*. Sequencing of the ITS region was used for molecular characterization and determination of these two isolates. ITS sequences from both cultures possessed 100% sequence identity to ITS sequences from reference strains of *A. apis* (GQ867766, U68313, KJ158165, KM242589, KM242591, KM242592, KT373974, MH859367) as found by the BLAST analysis conducted on the NCBI website (https://www.ncbi.nlm.nih.gov/, accessed on 2 November 2020). Additionally, the result of phylogenetic analysis performed using Bayesian methodology in Geneious software clustered these stains into a single clade, along with eight reference *A. apis* strains (Figure 6).

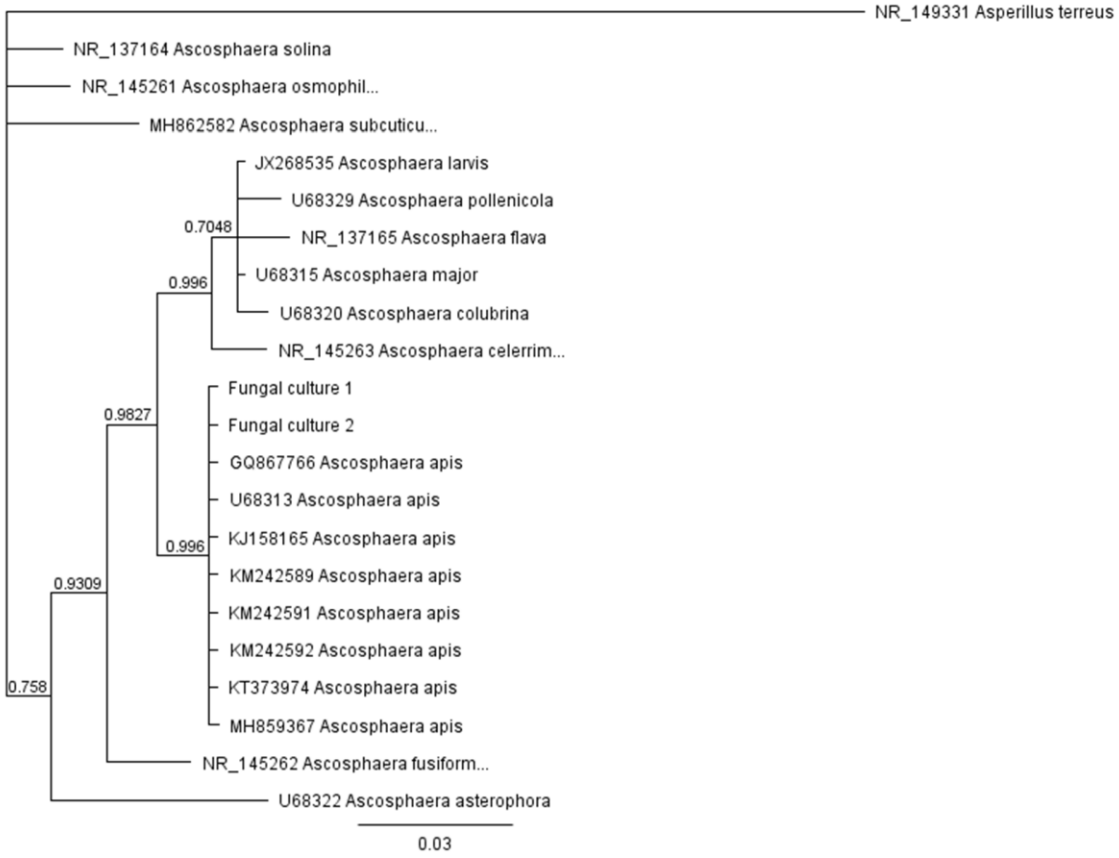


Figure 6. Phylogeny of the ITS region for selected *Ascosphaera species*. The phylogeny was inferred under Bayesian methodology in MrBayes using Geneious software. MrBayes was run for 100,000 generations with 50,000 sample points. For major phylogram branches, Bayesian posterior probabilities are shown. Taxa denoted as Fungal culture 1 and Fungal culture 2 represent sequence data generated in this study. These strains were isolated from mummified larvae, sequenced, and compared with other *Ascosphaera species*; their sequences as well as sequences for the outgroup (*Aspergillus terreus*) were obtained from GenBank.

3.1. Phylogeny of the ITS Region

We isolated two fungal strains from mummified honey bee larvae which were morphologically determined as *A. apis*. Sequencing of the ITS region was used for molecular characterization and determination of these two isolates. ITS sequences from both cultures possessed 100% sequence identity to ITS sequences from reference strains of *A.*

apis as found by the BLAST analysis conducted on the NCBI website. Additionally, the result of phylogenetic analysis performed using Bayesian methodology in Geneious software clustered these stains into a single clade, along with eight reference *A. apis* strains (Figure 6).

3.2. Growth Parameters

Both medium and temperature influence the growth rate of *A. apis* (Appendix A, Table A1, Figure 7). The growth is very close to the linear model (Appendix A, Tables A2 and A3, Figure 8). Both mating types grew fastest at 30 °C on each artificial medium on the 4th day of growth, except the low-nutrient medium CDA (Figure 7), on which *A. apis* grew faster at 25 °C. Mating type (−) grew faster than mating type (+) on almost every artificial medium. The exceptions are the low-nutrient media such as CDA and Oxoid ISA (Figure 7). After six days of growth, the mating type (+) of *A. apis* did not reach the edge of any of the media plates at any temperature. The fastest growth of the (+) mating type was recorded on PDA-BB8 (Day 6: 25 °C—69.75 mm, 30 °C—80.75 mm, 35 °C—70.00 mm) and YGPSA (Day 6: 25 °C—58.00 mm, 30 °C—79.50 mm, 35 °C—79.00 mm). However, the mycelium of mating type (−) reached the edge of the Petri dishes in 11/30 different variations (25 °C—PDA-BB4, 30 °C—SDA, PDAY, MEA, YGPSA, PDA-BB4, PDA-BB8, PDA-BB12, 35 °C—SDA, YGPSA, PDA-BB4) in six days. The best artificial media for fast growth of both mating types are YGPSA, PDA-BB12, PDA-BB8, SDA, and PDA-BB4. MEA and PDAY are also very suitable media. On the contrary, the growth on medium CDA was insufficient. As the fungal cultures reached the edge of the Petri dishes on the fifth day, statistical analyses were performed with the data of the fourth day of growth.

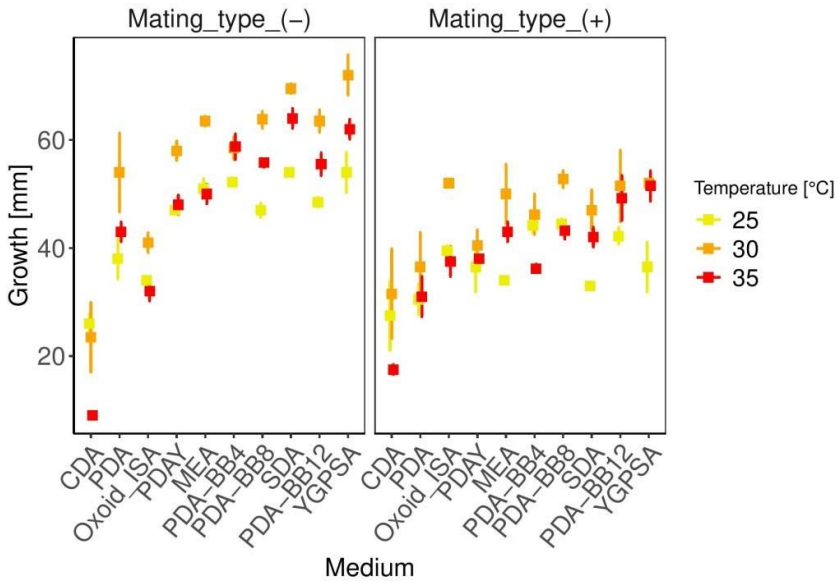


Figure 7. Effect of medium and temperature on the growth of both mating types of *A. apis* (4th day of growth). The error bars represent 95% confidence intervals.

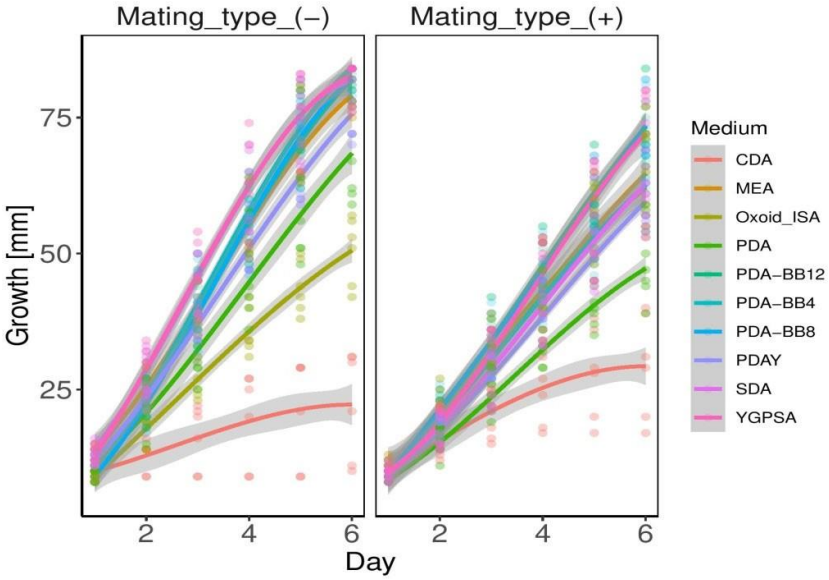


Figure 8. Scatter plot showing colony radial growth within six days, modeled using the cubic polynomial function. The regression lines were fitted by a generalized linear model with Poisson error distribution and link function log. The error lines represent 95% confidence intervals.

3.3. Production of Reproductive Structures

The number of reproductive structures, consisting of spore cysts, spore balls, and ascospores, was counted depending on the artificial media and temperature. Since ascospores are formed in spore balls and located in spore cysts, the reproductive structures were counted simultaneously to assess suitable conditions for the maturation and release of ascospores and spore balls. The production of reproductive structures is influenced by the artificial media and temperature (Appendix A, Table A4). The results are shown in Figure 9.

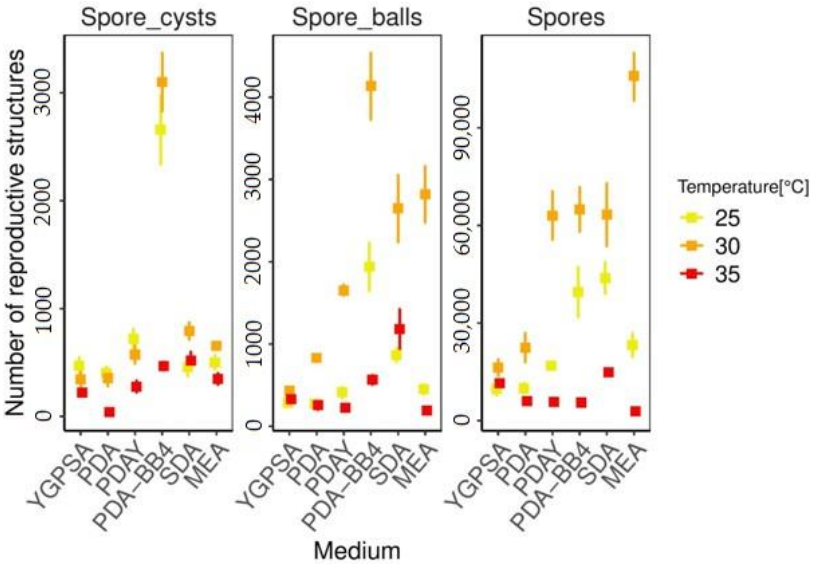


Figure 9. The mean number of reproductive structures (spore cysts, spore balls, and spores) per mm^2 on different culture media in combination with temperature. The error bars represent 95% confidence intervals.

3.3.1. Spore Cysts

For spore cyst production, both temperatures, 25 °C and 30 °C, are suitable. The temperature 35 °C is not suitable for high spore cyst production. The highest spore cyst production was recorded on the PDA-BB4 medium at 30 °C (3101/mm^2), which was 4–9-fold higher than on the other media (30 °C—SDA: 792/mm^2, MEA: 647/mm^2, PDAY: 582/mm^2, PDA: 356/mm^2, YGPSA: 343/mm^2). Thus, the addition of bee brood to an artificial medium has a significant effect on the fungus's sexual reproduction.

3.3.2. Spore Balls

Production of spore balls was also higher at 30 °C on all the tested media, especially on PDA-BB4 (4338/mm^2), MEA (2822/mm^2), and SDA (2645/mm^2), which correlated with the number of spore cysts produced.

3.3.3. Ascospores

Though the most ascospores were observed on MEA agar at 30 °C (99,245/mm^2), the lowest number of ascospores was also produced on the MEA agar (2797/mm^2), at 35 °C. Therefore, the appropriate temperature setting is crucial to ensure high ascospore production. Very high ascospore concentrations were also detected on PDA-BB4 (64,900/mm^2), SDA (63,252/mm^2), and PDA+Y (63,127/mm^2), all at 30 °C.

3.4. Morphometry of Reproductive Structures

The temperature and artificial media affect the size of the reproductive structures of *A. apis* (Appendix A, Table A5). The results imply that, in more suitable media and temperatures, *A. apis* produce smaller spore cysts and bigger spore balls and vice versa (Figure 10). The biggest spore cysts were observed at 25 °C with the exception of SDA, where the result is not statistically significant. On average, the largest spore cysts were produced on SDA (25 °C—82.45 µm, 30 °C—75.28 µm, 35 °C—79.75 µm) and MEA (25 °C—83.75 µm, 30 °C—73.29 µm, 35 °C—77.54 µm). The smallest spore cysts were produced on PDA (25 °C—76.44 µm, 30 °C—71.08 µm, 35 °C—67.23 µm). This may also correlate with the amount of nutrients in the media.

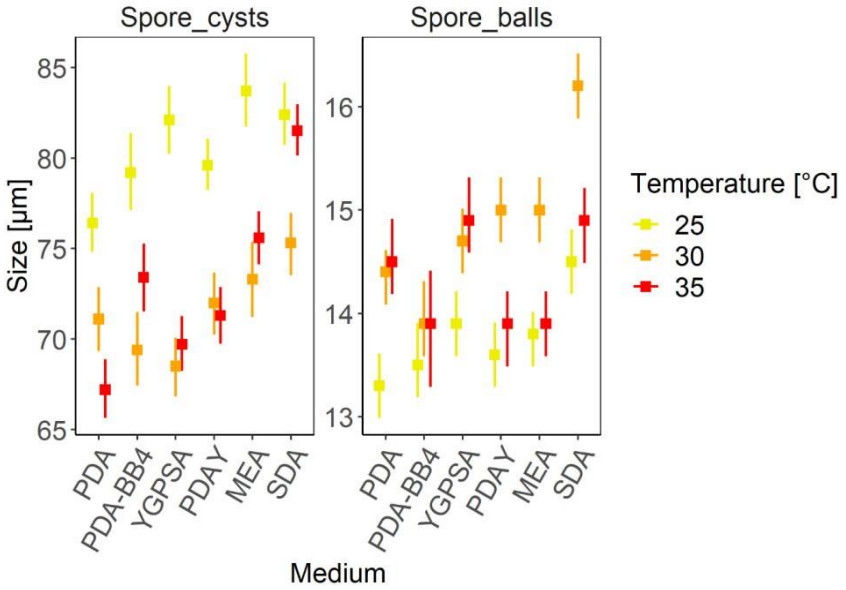


Figure 10. The mean size of reproductive structures (spore cysts and spore balls) in different culture media, in combination with temperature. The error bars represent 95% confidence intervals.

The opposite situation is true of the spore ball size. Bigger spore balls were produced at a suitable temperature. If the temperature changed from the optimum, the size was smaller. On most of the tested media, spore balls were significantly bigger at 30 °C in comparison with 25 °C with the exception of PDA-BB4. In some cases (PDAY, MEA, SDA), the spore balls at 30 °C were bigger than at 35 °C. Overall, the largest spore balls were produced on SDA (30 °C = 16.21 ± 2.19 μm).

4. Discussion

4.1. Growth Parameters and Optimal Temperature

Ascosphaera apis grew faster on sugar-rich media such as SDA, YGPSA, MEA, PDA-Y, or PDA-BB. Slower growth was recorded on sugarless media such as CDA and Oxoid ISA (Figure 7). This is in agreement with Heath [58], who stated that *A. apis* grows very well on sugar-rich media because it can easily utilize arabinose, dextrose, mannose, galactose, sucrose, maltose, lactose, trehalose, glucose, fructose, and even dextrin and starch. On the contrary, high-fat and high-nitrogen media are not suitable for *A. apis* growth [59].

The optimal temperature for *A. apis* cultivation on commonly used artificial media was 30 °C for both mating types, (+) and (−). This could be one of the reasons why *A. apis* spreads faster in weak bee colonies, which are unable to maintain a high and stable temperature in beehives. Nevertheless, Flores et al. [37] reported the highest rate of infection in bee brood kept in vitro at 25 °C (77.62%), less at 30 °C (15.31%), and the lowest rate at 35 °C (2.22%). These differences between the growth of *A. apis* on artificial media and in natural conditions are probably caused by the low temperature for the development of bee brood. The lower than optimal temperature reduces the activity of physiological processes and contributes to a slower metabolism of bee brood, which makes bee brood more susceptible to pathogens [60,61].

Additionally, other studies reported an optimal *A. apis* growth rate near 30 °C [21,62,63]. However, *A. apis* is able to grow in a broad temperature range, from 22 °C [64] to 45 °C [65]. Therefore, the optimal growth conditions seem to depend on the particular strain and area of its occurrence.

Claussen [66] and Maurizio [63] stated that the male mating type (−) grows faster than the female mating type (+), which correlates with the results of this study. However, Evison et al. [67], Bissett [64], and Anderson and Gibson [62] did not notice any differences depending on the growth of opposite mating types. Claussen [66] and Bissett [64] also found yellowish pigment in the (−) mating type. The yellowish color was observed in this study as well; however, this phenomenon was not observed by Spiltoir [16]. Growth differences between opposite mating types probably depend on the particular *A. apis* strain, and the yellowish pigment could be caused by faster aging due to a lack of nutrients.

Since the male mating type (−) grows faster than the female mating type (+), it usually also requires more nutrients for its development due to a faster metabolism [68]. That is the reason for faster consumption of all nutrients from media and consequently the earlier aging and changing color. Low-nutrient media contain an insufficient amount of nutrients, so the generally faster-growing mating types are restricted. On the other hand, the slower-growing mating type can better exploit lower-nutrient media and, under those conditions, fares better (Figure 7).

Commercially available media can be improved by protein supplements. The addition of yeast to PDA medium promoted the growth of *A. apis*. Even better results were achieved by the addition of mixed bee brood to PDA (Figure 7, Appendix A, Table A3). The sufficient amount of bee brood to ensure faster fungal growth is 40 g/L media. In general, nutrient-rich media produced better results compared to the nutrient-poor media [47].

4.2. Reproductive Structure Production

To assess the reproductive structure development, the spore cysts, spore balls, and ascospores were counted simultaneously. The method does not allow for counting the real number of ascospores; instead, it involves assessing the natural level of maturation and release of spore balls and ascospores depending on the media and temperature. According to our results, both media and temperature affect ascospore release (Appendix A, Table A4), and thus a lot of immature ascospores can remain for different lengths of time in spore balls or cysts. Ruffinengo et al. [69] only counted ascospores after the stirring of the spore cysts. However, that method may be inaccurate because counting the real number of ascospores is very difficult as not every ascospore is properly released from reproductive structures.

In general, the more suitable the medium and temperature for *A. apis* development, the more ascospores and spore balls will mature and be released in a shorter period of time and vice versa. At 25 °C, more spore cysts than spore balls were observed on each medium (Figure 9), which indicated slow reproductive structure development and maturity because of the lack of ascospores and spore balls released. However, at 30 °C, significantly more ascospores and spore balls were released. The lowest reproductive structure release rate was recorded at 35 °C due to less favorable conditions. In addition, if there is less suitable medium, spore cysts crumble very little and only a small number of spore balls and ascospores are released. Ascospore maturation and release are heavily influenced by the temperature. For example, the lowest and highest ascospore concentrations were observed on MEA at 35 °C (2797/mm^2) and 30 °C (99,245/mm^2), respectively. However, since *A. apis* produced the greatest number of spore cysts and spore balls on the PDA-BB4 medium, this medium has the potential to produce the greatest number of ascospores as well. In this case, the ascospore maturation takes longer than on MEA agar, indicating that malt extract accelerates maturation. Nevertheless, Ruffinengo et al. [69] did not find a significant effect of the culture medium on the number of produced ascospores. This was likely due to the different media composition compared to this study.

In general, the highest spore yields are obtained in media with a C/N ratio of 10:1, which could represent potato dextrose agar (PDA) [50]. However, in this experiment, *A. apis* reproductive structure production was lower on PDA at the optimal temperature (30 °C) in comparison to the other tested media. These parameters were improved only after the addition of yeast (PDAY), but especially by honey bee brood supplementation (Figures 7–9).

The number of produced ascospores can vary greatly, even under the same conditions. Ruffinengo et al. [69] reported only 7.42 ascospores per 1 mm^2 on PDA at 30 °C, but we recorded 22,410 ascospores per 1 mm^2 in the same conditions. The ascospore production could have been affected by a particular strain and also by the height of mycelium in the Petri dishes where the ascospores were produced. On most of our tested media, the mycelium grew to the top of the lid. Therefore, reproductive structures were produced in the whole column of mycelium. At the time of sample collection, most of the mycelia were degraded and the number of reproductive structures per area increased. The other issue is the area of the Petri dishes where the samples were taken from, because the sporulated area can differ in size. In some cases, the sporulated area can be thinner than the diameter of the cork borer. The bigger the cork borer that is used, the fewer reproductive structures are counted in the average 1 mm^2 area. In this study, the sporulated area covered the whole cork borer diameter. That could be the main cause of the differences in the abovementioned results. Other authors reported that the mean value of ascospores on a single mummy varies between 10^4 and 10^9 depending on bee mummy color, which is affected by loads of spore cysts [70,71], which correlates with our observations.

4.3. Morphometry of Reproductive Structures

This study confirmed that temperature and artificial media affect the size of spore cysts and spore balls (ANOVA, $F_{(10, 228)} = 7.5380$, $p < 0.001$) (Appendix A, Table A5). Ascospores are almost unaffected by media and temperature [69] and too small to measure precisely. For that reason, ascospore morphometry was not included in this study.

During the measurement of spore cyst and spore ball size, a specific pattern was found. In less suitable conditions, large spore cysts and small spore balls were observed, and vice versa (Figure 10). This means that, for example, *A. apis* growing on SDA at 30 °C (suitable conditions) produced relatively small spore cysts and big spore balls. On the contrary, *A. apis* growing on PDA at 25 °C (less suitable conditions) produced relatively big spore cysts and small spore balls (Figure 10). The reason could be that, in suitable conditions, *A. apis* does not need to create big and strong cysts to protect ascospores, instead investing energy into creating bigger spore balls, which means more ascospores as a result. On the contrary, in worse conditions, it is preferable to create big and strong cysts, which help ascospores to survive an unfavorable period of time. This phenomenon can also be observed when compared with two other studies from Argentina. While the authors examined spore cysts in unfavorable conditions on solitary bees *Xylocopa augusti* [72], the average size of spore cysts was significantly bigger than the spore cysts produced under suitable conditions on artificial media [69]. In addition, the solitary bees' brood is exposed to fluctuating temperatures, which could be the main reason for the greater spore cyst production. In former studies, smaller spore balls (12.5 μm, 12.0 μm, 12.1 μm) and bigger spore cysts (80.2 μm, 70.0 μm, 84.5 μm) were found at lower temperatures [62,64,73], while bigger spore balls (up to 16 μm) and smaller spore cysts (50–60 μm) were found at 30 °C [23]. The pattern can be seen in all the tested media in this study. On average, *A. apis* produced the smallest spore cysts and biggest spore balls at 30 °C. Only in the case of PDA were the spore cysts smallest at 35 °C, which indicates a more suitable cultivation temperature on that medium at 35 °C. If the sizes of spore cysts created at 30 °C and 35 °C are not significantly different, the optimal cultivation temperature seems to be between these values. The exceptions are spore balls produced on PDA-BB4, the size of which was not affected by the temperature. The addition of bee brood could simulate natural conditions and eliminate the effect of temperature on the spore ball morphometry, which is evident in artificial conditions.

However, the size of reproductive structures can also differ considerably depending on the strain. That is obvious when we compare the abovementioned Argentinian strain and the strain used in this study, cultivated under the same conditions (PDA at 30 °C). In addition, the size of the spore balls of the Argentinian strain was not significantly affected by the culture media. That is not in agreement with the results of this study (ANOVA, $F_{(5, 228)} = 33.7521$, $p < 0.001$). The sizes of the reproductive structures were very variable and depended on the temperature and artificial media. For example, on PDA at 35 °C, the mean spore cyst size was only 67.22 ± 12.17 μm; however, on MEA at 25 °C, the mean spore cyst size was 83.75 ± 15.22 μm. Spore balls are also affected by media and temperature. On PDA at 25 °C and SDA at 30 °C, the mean spore ball sizes were 13.32 ± 2.27 μm and 16.21 ± 2.19 μm, respectively.

4.4. Strain Competition

The growth rate of *Ascosphaera apis* mating types, also representing the degree of virulence, is very important because of the superinfection model of evolution in which strains do not cooperate but rather compete with each other [67]. If there is only one parasite invading a host, it can fully exploit the host nutrients because of an absence of competition [74]. However, if the host is attacked by more parasites, which is very common in nature, even with *A. apis* [75], it is very likely that less virulent parasites will be outcompeted or suppressed by the faster-growing ones. That leads to the selection of more virulent strains [76], assuming a high transmission ability [77]. Evison et al. [67]

claim that more virulent *A. apis* strains may have an evolutionary advantage. They observed an increase in virulence after artificial co-infection by several strains in three generations. Some of the replicate lines of the parasite even disappeared. The model of *Ascosphaera* superinfection has also been observed by Klinger et al. [78], who tested three *Ascosphaera* species in a mixed infection. However, increasing virulence often leads to a decrease in parasite fitness because of faster host death and fewer nutrients [77]. It is likely that, after some time, only the most effectively host-utilized strains will remain in an area. The very high- or low-virulence strains will disappear [67].

An exception would be, for example, a heterothallic species, where two opposite mating types are needed to ensure reproduction. In this case, there may even be an advantage for a less virulent and less abundant mating type [79,80]. The evolutionary advantage of slower-growing mating types is better competitiveness in specific situations—for example, they can better exploit poor nutrient sources because of their slow growth. On the contrary, a more virulent mating type needs more nutrients to support its faster growth. It is demonstrated in this paper that only on poor-nutrient media (CDA and Oxoid ISA) did the (+) mating type grow faster than the (−) mating type (Figure 7). Another advantage lies in the low reproduction limitation because a faster-growing mating type is probably more abundant. That means it is easier to mate and survive in a given area.

Reproduction with a slower-growing mating type can explain the occurrence of white bee brood mummies, which are supposed to be without ascospores; however, Gochnauer and Margetts [71] found a few spore cysts on the surface of white mummies. This indicates the presence of both mating types in the mummy, but the faster-growing one may have prevailed and consumed all nutrients from the host. In general, a more virulent mating type prevails and outcompetes a slower one in a body cavity; therefore, there is no or little sporulation on the surface of the mummy. White mummies can occur often and in significant amounts. Gilliam [81] observed 35% white mummies in his experiment. This could be a way of reducing ascospore loads in infected beehives, ensuring balanced parasite–host evolution.

5. Conclusions

The study provides a comprehensive summary of the cultivation conditions for culturing *A. apis* on artificial media and explains the biology and specific behavior pattern of the fungus depending on different conditions. The optimal temperature for the fungus cultivation is 30 °C because it allows the fastest growth, the highest reproductive structure production, the fastest release, and the greatest spore ball formation. The results of the study clarify the genesis of reproductive structures depending on the conditions and confirm the importance of the bee brood for the pathogen's high reproduction rate. The article also gives a possible explanation of the occurrence of pure white chalkbrood mummies, which is based on the different growth rates of individual strains of *A. apis.* This mechanism balances the parasite–host relationship in nature. These results can be used in subsequent studies, which are needed to gain a better understanding of *A. apis* pathogenesis and virulence, especially in the conditions of a live bee brood.

Author Contributions: Conceptualization, P.M.; data curation, J.Š. and M.R.; formal analysis, A.B., J.K., and P.K.; funding acquisition, P.K.; investigation, P.M. and I.H.; methodology, P.M. and A.B.; resources, P.K.; supervision, A.B. and V.Č.; writing—original draft, P.M., M.H., and M.K. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the University of South Bohemia in Ceske Budejovice, research project GAJU 059/2019/Z, GAJU 027/2019/Z and NAZV QK 1910356.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The study did not report any data.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

Appendix A

Table A1. Results of the aligned rank transformed ANOVA testing the effect of temperature, culture medium, and mating type on the radial growth of *A. apis* (4th day of growth).

Explanatory Variables	F	Df.	Df. Residual	*p*-Value
Medium	21.204	9	180	<0.001
Temperature	16.370	2	180	<0.001
Mating type	38.523	1	180	<0.001
Medium:Temperature	0.956	18	180	0.5121
Medium:Mating type	4.591	9	180	<0.001
Temperature:Mating type	0.096	2	180	0.9082
Medium:Temperature:Mating type	0.096	18	180	0.9982

Table A2. Results of generalized estimating equation approach, testing the effect of temperature, culture medium, day (repeated measurements in time), and mating type on the radial colony growth.

Explanatory Variables	Df.	χ^2	*p*-Value
Medium	9	374.1	<0.001
Day	1	6724.2	<0.001
Mating type	1	290.0	<0.001
Temperature	2	297.5	<0.001
Medium:Day	9	81.8	<0.001
Medium:Mating type	9	423.6	<0.001
Day:Mating type	1	2.7	0.098
Medium:Day:Mating type	9	14.0	0.122

Table A3. Regression slopes estimated by the generalized estimating equation approach, testing the relationship between *A. apis* growth on different culture media within six days; mating type and temperature were used in the model as covariates.

Medium	Regression Slopes	Standard Error	*p*-Value
YGPSA	2.53	0.06857	<0.001
PDA-BB12	2.49	0.06282	<0.001
PDA-BB8	2.48	0.06392	<0.001
SDA	2.47	0.06833	<0.001
PDA-BB4	2.45	0.05889	<0.001
MEA	2.45	0.06612	<0.001
PDAY	2.36	0.06224	<0.001
PDA	2.22	0.06724	<0.001
Oxoid ISA	2.23	0.07234	<0.001
CDA	1.66	0.08585	<0.001

Table A4. Results of the aligned rank transformed ANOVA, testing the effect of temperature and culture medium on the production of different reproductive structures per mm^2.

Explanatory Variables	F	Df.	Df. Residual	*p*-Value
Medium	314.91	5	162	<0.001
Temperature	1128.41	2	162	<0.001
Rep. structures	1549.62	2	162	<0.001
Medium:Temperature	183.04	10	162	<0.001
Medium:Rep. structures	235.06	10	162	<0.001
Temperature:Rep. structures	1268.48	4	162	<0.001
Medium:Temperature:Rep. structures	124.21	20	162	<0.001

Table A5. Results of the aligned rank transformed ANOVA, testing the effect of temperature and culture medium on the size of different reproductive structures.

Explanatory Variables	F	Df.	Df. Residual	*p*-Value
Medium	33.7521	5	228	<0.001
Temperature	85.8862	2	228	<0.001
Rep. structures	6794.2763	1	228	<0.001
Medium:Temperature	6.0024	10	228	<0.001
Medium:Rep. structures	24.3856	5	228	<0.001
Temperature:Rep. structures	127.0338	2	228	<0.001
Medium:Temperature:Rep. structures	7.5380	10	228	<0.001

References

1. Gallai, N.; Salles, J.-M.; Settele, J.; Vaissière, B.E. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecol. Econ.* **2009**, *68*, 810–821.
2. Kearns, C.A.; Inouye, D.W.; Waser, N.M. ENDANGERED MUTUALISMS: The Conservation of Plant-Pollinator Interactions. *Annu. Rev. Ecol. Syst.* **1998**, *29*, 83–112, doi:10.1146/annurev.ecolsys.29.1.83.
3. Klein, A.-M.; Vaissiere, B.E.; Cane, J.H.; Steffan-Dewenter, I.; Cunningham, S.A.; Kremen, C.; Tscharntke, T. Importance of pollinators in changing landscapes for world crops. *Proc. R. Soc. B Biol. Sci.* **2007**, *274*, 303–313.
4. Aizen, M.A.; Garibaldi, L.A.; Cunningham, S.A.; Klein, A.M. How much does agriculture depend on pollinators? Lessons from long-term trends in crop production. *Ann. Bot.* **2009**, *103*, 1579–1588, doi:10.1093/aob/mcp076.
5. Biesmeijer, J.C.; Roberts, S.P.; Reemer, M.; Ohlemüller, R.; Edwards, M.; Peeters, T.; Schaffers, A.; Potts, S.G.; Kleukers, R.; Thomas, C. Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science* **2006**, *313*, 351–354.
6. Oldroyd, B.P. What's killing American honey bees? *PLoS Biol.* **2007**, *5*, e168.
7. Stokstad, E. The case of the empty hives. *Science* **2007**, *316*, 970–972.
8. Yoder, J.A.; Jajack, A.J.; Cornacchione, W.S.; Dunn, A.L.; Cunningham, E.G.; Matchett, C.L.; Rosselot, A.E. In vitro evaluation of sugar syrups, antibiotics, and miticides on growth of honey bee pathogen, *Ascosphaera apis*: Emphasis for chalkbrood prevention is on keeping bees healthy. *Apidologie* **2014**, *45*, 568–578.
9. Klein, S.; Cabirol, A.; Devaud, J.-M.; Barron, A.B.; Lihoreau, M. Why bees are so vulnerable to environmental stressors. *Trends Ecol. Evol.* **2017**, *32*, 268–278.
10. Sandrock, C.; Tanadini, M.; Tanadini, L.G.; Fauser-Misslin, A.; Potts, S.G.; Neumann, P. Impact of chronic neonicotinoid exposure on honeybee colony performance and queen supersedure. *PLoS ONE* **2014**, *9*, e103592.
11. Alaux, C.; Ducloz, F.; Crauser, D.; Le Conte, Y. Diet effects on honeybee immunocompetence. *Biol. Lett.* **2010**, *6*, 562–565.
12. Hybl, M.; Mraz, P.; Sipos, J.; Pridal, A. Effects of phenolic bioactive substances on reducing mortality of bees (*Apis mellifera*) intoxicated by thiacloprid. In Proceedings of the MendelNet, Brno, Czech Republic, 6–7 November 2019; pp. 131–135.
13. Fitzpatrick, Ú.; Murray, T.E.; Paxton, R.J.; Breen, J.; Cotton, D.; Santorum, V.; Brown, M.J. Rarity and decline in bumblebees–a test of causes and correlates in the Irish fauna. *Biol. Conserv.* **2007**, *136*, 185–194.
14. Ansari, M.J.; Al-Ghamdi, A.; Usmani, S.; Khan, K.A.; Alqarni, A.S.; Kaur, M.; Al-Waili, N. In vitro evaluation of the effects of some plant essential oils on *Ascosphaera apis*, the causative agent of Chalkbrood disease. *Saudi J. Biol. Sci.* **2017**, *24*, 1001–1006.
15. Higes, M.; Martín-Hernández, R.; Garrido-Bailón, E.; González-Porto, A.V.; García-Palencia, P.; Meana, A.; Del Nozal, M.J.; Mayo, R.; Bernal, J.L. Honeybee colony collapse due to Nosema ceranae in professional apiaries. *Environ. Microbiol. Rep.* **2009**, *1*, 110–113.
16. Spiltoir, C.F. Life cycle of *Ascosphaera apis* (*Pericystis apis*). *Am. J. Bot.* **1955**, *42*, 501–508.
17. Strasser, H. Use of hyphomycetous fungi for managing insect pests. In *Fungi as Biocontrol Agents: Progress Problems and Potential*; CABI: Wallingford, UK, 2001; p. 23.
18. Mannino, M.C.; Huarte-Bonnet, C.; Davyt-Colo, B.; Pedrini, N. Is the insect cuticle the only entry gate for fungal infection? Insights into alternative modes of action of entomopathogenic fungi. *J. Fungi* **2019**, *5*, 33.
19. James, T.Y.; Kauff, F.; Schoch, C.L.; Matheny, P.B.; Hofstetter, V.; Cox, C.J.; Celio, G.; Gueidan, C.; Fraker, E.; Miadlikowska, J. Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature* **2006**, *443*, 818–822.
20. Heath, L.; Gaze, B.M. Carbon dioxide activation of spores of the chalkbrood fungus *Ascosphaera apis*. *J. Apic. Res.* **1987**, *26*, 243–246.
21. Bailey, L. The effect of temperature on the pathogenicity of the fungus, *Ascosphaera apis*, for larvae of the honey bee, *Apis mellifera*. *Insect Pathol. Microb. Control* **1966**, 162–167.
22. Aronstein, K.A.; Murray, K.D. Chalkbrood disease in honey bees. *J. Invertebr. Pathol.* **2010**, *103*, S20–S29.
23. Li, Z.; You, X.-L.; Wang, L.-L.; Yan, Z.-T.; Zhou, Z.-Y. Spore morphology and ultrastructure of an Ascosphaera apis strain from the honeybees (*Apis mellifera*) in southwest China. *Mycologia* **2018**, *110*, 325–338.
24. Liu, T.; Peng, C.; Mussen, E.; Marston, J.; Munn, R. Ultrastructure of the freeze-etched spore of Ascosphaera apis, an entomopathogenic fungus of the honeybee *Apis mellifera*. *J. Invertebr. Pathol.* **1991**, *57*, 371–379.

25. Bamford, S.; Heath, L. The effects of temperature and pH on the germination of spores of the chalkbrood fungus, Ascosphaera apis. *J. Apic. Res.* **1989**, *28*, 36–40.
26. Jensen, A.B.; Aronstein, K.; Flores, J.M.; Vojvodic, S.; Palacio, M.A.; Spivak, M. Standard methods for fungal brood disease research. *J. Apic. Res.* **2013**, *52*, 1–20.
27. Evison, S.E. Chalkbrood: Epidemiological perspectives from the host–parasite relationship. *Curr. Opin. Insect Sci.* **2015**, *10*, 65–70.
28. Heath, L. Development of chalk brood in a honeybee colony: A review. *Bee World* **1982**, *63*, 119–130.
29. De Jong, D. Experimental enhancement of chalk brood infections. *Bee World* **1976**, *57*, 114–115.
30. Hedtke, K.; Jensen, P.M.; Jensen, A.B.; Genersch, E. Evidence for emerging parasites and pathogens influencing outbreaks of stress-related diseases like chalkbrood. *J. Invertebr. Pathol.* **2011**, *108*, 167–173.
31. Vojvodic, S.; Jensen, A.B.; James, R.R.; Boomsma, J.J.; Eilenberg, J. Temperature dependent virulence of obligate and facultative fungal pathogens of honeybee brood. *Vet. Microbiol.* **2010**, *149*, 200–205.
32. Dallman, H. Neue Wege bei der Bekämpfung der Kalkbrut in Bienenenvölkern. *Merkbl. Imker Verb. KleingärtnerSiedl. Klein-Tierzüchter* **1974**, *8*, 1–36.
33. Dreher, K. Auftreten von Bienenkrankheiten in Niedersachsen und Braunschweig im Jahre 1937. *Niedersachs. Imker* **1938**, *73*, 282–284.
34. Roussy, L. Nouvelle contribution à l'étude du Per-icystis apis. *Gaz Apic* **1962**, *63*, 101–105.
35. Cooper, B. Fluctuating broodnest temperature rhythm. *Br. Isles Bee Breed. News* **1980**, *18*, 1216.
36. Tabarly, O.; Monteira, E. Influence de la teneur en eau du miel sur le développement des mycoses. *Bull Apic.* **1961**, *4*, 31–34.
37. Flores, J.; Ruiz, J.; Ruz, J.; Puerta, F.; Bustos, M.; Padilla, F.; Campano, F. Effect of temperature and humidity of sealed brood on chalkbrood development under controlled conditions. *Apidologie* **1996**, *27*, 185–192.
38. Koenig, J.; Boush, G.M.; Erickson, E., Jr. Effects of spore introduction and ratio of adult bees to brood on chalkbrood disease in honeybee colonies. *J. Apic. Res.* **1987**, *26*, 191–195.
39. Chantawannakul, P.; Puchanichanthranon, T.; Wongsiri, S. Inhibitory effects of some medicinal plant extracts on the growth of Ascosphaera apis. In *III WOCMAP Congress on Medicinal and Aromatic Plants—Volume 4: Targeted Screening of Medicinal and Aromatic Plants, Economics 678*; ISHS: Chiang Mai, Thailand, 2005; pp. 183–189.
40. Chen, D.; Guo, R.; Xiong, C.; Zheng, Y.; Hou, C.; Fu, Z. Morphological and molecular identification of chalkbrood disease pathogen Ascosphaera apis in Apis cerana cerana. *J. Apic. Res.* **2018**, *57*, 516–521.
41. Zaghloul, O.; Mourad, A.; El Kady, M.B.; Nemat, F.; Morsy, M. Assessment of losses in honey yield due to the chalkbrood disease, with reference to the determination of its economic injury levels in Egypt. *Commun. Agric. Appl. Biol. Sci.* **2005**, *70*, 703–714.
42. Guo, R.; Chen, D.; Xiong, C.; Hou, C.; Zheng, Y.; Fu, Z.; Diao, Q.; Zhang, L.; Wang, H.; Hou, Z. Identification of long non-coding RNAs in the chalkbrood disease pathogen *Ascospheara apis*. *J. Invertebr. Pathol.* **2018**, *156*, 1–5.
43. Gilliam, M.; TABER, S., III; Richardson, G.V. Hygienic behavior of honey bees in relation to chalkbrood disease. *Apidologie* **1983**, *14*, 29–39.
44. Chaimanee, V.; Thongtue, U.; Sornmai, N.; Songsri, S.; Pettis, J.S. Antimicrobial activity of plant extracts against the honeybee pathogens, Paenibacillus larvae and *Ascosphaera apis* and their topical toxicity to Apis mellifera adults. *J. Appl. Microbiol.* **2017**, *123*, 1160–1167.
45. Mraz, P.; Bohata, A.; Hostickova, I.; Kopecky, M.; Zabka, M.; Hybl, M.; Curn, V. Inhibitory effect of selected botanical compounds on the honey bee fungal pathogen *Ascosphaera apis*. In Proceedings of the MendelNet, Brno, Czech Republic, 6–7 November 2019; pp. 474–479.
46. Vega, F.E.; Goettel, M.S.; Blackwell, M.; Chandler, D.; Jackson, M.A.; Keller, S.; Koike, M.; Maniania, N.K.; Monzon, A.; Ownley, B.H. Fungal entomopathogens: New insights on their ecology. *Fungal Ecol.* **2009**, *2*, 149–159.
47. Ibrahim, L.; Butt, T.M.; Jenkinson, P. Effect of artificial culture media on germination, growth, virulence and surface properties of the entomopathogenic hyphomycete Metarhizium anisopliae. *Mycol. Res.* **2002**, *106*, 705–715.
48. Liu, F.; Wu, X.; He, Z.; Xiang, M.; He, Y.; Liu, X.; Chen, S.; Zeng, W. Nutritional effects on the mycelial growth and enzymatic activity of *Isaria farinosa*, and *Hepialus larvae* growth. *J. Appl. Microbiol.* **2016**, *120*, 1605–1615.
49. Wyss, G.S.; Charudattan, R.; DeValerio, J.T. Evaluation of agar and grain media for mass production of conidia *of Dactylaria higginsii*. *Plant Dis.* **2001**, *85*, 1165–1170.
50. Safavi, S.; Shah, F.A.; Pakdel, A.K.; Reza Rasoulian, G.; Bandani, A.R.; Butt, T.M. Effect of nutrition on growth and virulence of the entomopathogenic fungus *Beauveria bassiana*. *FEMS Microbiol. Lett.* **2007**, *270*, 116–123.
51. Garrido-Bailón, E.; Higes, M.; Martínez-Salvador, A.; Antúnez, K.; Botías, C.; Meana, A.; Prieto, L.; Martín-Hernández, R. The prevalence of the honeybee brood pathogens *Ascosphaera apis, Paenibacillus larvae* and *Melissococcus plutonius* in S panish apiaries determined with a new multiplex PCR assay. *Microb. Biotechnol.* **2013**, *6*, 731–739.
52. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403–410.
53. Sayers, E.W.; Barrett, T.; Benson, D.A.; Bolton, E.; Bryant, S.H.; Canese, K.; Chetvernin, V.; Church, D.M.; DiCuccio, M.; Federhen, S. Database resources of the national center for biotechnology information. *Nucleic Acids Res.* **2010**, *39*, D38–D51.
54. Wobbrock, J.O.; Findlater, L.; Gergle, D.; Higgins, J.J. The aligned rank transform for nonparametric factorial analyses using only anova procedures. In Proceedings of the SIGCHI Conference on Human Factors in Computing Systems, Vancouver, BC, Canada, 7–12 May 2011; pp. 143–146.

55. Mansouri, H. Multifactor analysis of variance based on the aligned rank transform technique. *Comput. Stat. Data Anal.* **1998**, *29*, 177–189.
56. Lenth, R.; Singmann, H.; Love, J.; Buerkner, P.; Herve, M. Emmeans: Estimated marginal means, aka least-squares means. *R Package Version* **2018**, *1*, 3.
57. Yan, J.; Fine, J. Estimating equations for association structures. *Stat. Med.* **2004**, *23*, 859–874.
58. Heath, L. Chalk brood pathogens: A review. *Bee World* **1982**, *63*, 130–135.
59. Huber, J. Untersuchungen zur Physiologie insektentötender Pilze. *Arch. Für Mikrobiol.* **1958**, *29*, 257–276.
60. Medrzycki, P.; Sgolastra, F.; Bortolotti, L.; Bogo, G.; Tosi, S.; Padovani, E.; Porrini, C.; Sabatini, A.G. Influence of brood rearing temperature on honey bee development and susceptibility to poisoning by pesticides. *J. Apic. Res.* **2010**, *49*, 52–59.
61. Wang, Q.; Xu, X.; Zhu, X.; Chen, L.; Zhou, S.; Huang, Z.Y.; Zhou, B. Low-temperature stress during capped brood stage increases pupal mortality, misorientation and adult mortality in honey bees. *PLoS ONE* **2016**, *11*, e0154547.
62. Anderson, D.L.; Gibson, N.L. New species and isolates of spore-cyst fungi (Plectomycetes: Ascosphaerales) from Australia. *Aust. Syst. Bot.* **1998**, *11*, 53–72.
63. Maurizio, A. Beiträge zur Kenntnis der Pilzflora im Bienenstock: Die Pericystit-Infektion der Bienenlarven, *Ber. Schweiz. Bot. Ges.* **1935**, *44*, 133–156.
64. Bissett, J. Contribution toward a monograph of the genus Ascosphaera. *Can. J. Bot.* **1988**, *66*, 2541–2560.
65. Eble, H.; Weide, W. Die Bekämpfung von Pericystis apis Maassen in Labor und Praxis. *Wiss. Z. Univ. HalleMath. Nat. Riehe* **1961**, *10*, 83–86.
66. Claussen, P. *Entwicklungsgeschichtliche Untersuchungen über den Erreger der als" Kalkbrut" bezeichneten Krankheit der Bienen*; Parey: Berlin, Germany, 1921.
67. Evison, S.; Foley, K.; Jensen, A.; Hughes, W. Genetic diversity, virulence and fitness evolution in an obligate fungal parasite of bees. *J. Evol. Biol.* **2015**, *28*, 179–188.
68. Damialis, A.; Mohammad, A.B.; Halley, J.M.; Gange, A.C. Fungi in a changing world: Growth rates will be elevated, but spore production may decrease in future climates. *Int. J. Biometeorol.* **2015**, *59*, 1157–1167.
69. Ruffinengo, S.; Peña, N.; Clemente, G.; Palacio, M.; Escande, A. Suitability of culture media for the production of ascospores and maintenance of Ascosphaera apis. *J. Apic. Res.* **2000**, *39*, 143–148.
70. Flores, J.; Spivak, M.; Gutiérrez, I. Spores of Ascosphaera apis contained in wax foundation can infect honeybee brood. *Vet. Microbiol.* **2005**, *108*, 141–144.
71. Gochnauer, T.; Margetts, V. Decontaminating effect of ethylene oxide on honeybee larvae previously killed by chalk-brood disease. *J. Apic. Res.* **1980**, *19*, 261–264.
72. Reynaldi, F.J.; Lucia, M.; Garcia, M.L.G. *Ascosphaera apis*, the entomopathogenic fungus affecting larvae of native bees (Xylocopa augusti): First report in South America. *Rev. Iberoam. Micol.* **2015**, *32*, 261–264.
73. Chorbinski, P.; Rypula, K. Studies on the morphology of strains *Ascosphaera apis* isolated from chalkbrood disease of the honey bees. *Electron. J. Pol. Agric. Univ.* **2003**, *6*, 1–12.
74. Frank, S.A. Models of parasite virulence. *Q. Rev. Biol.* **1996**, *71*, 37–78.
75. Gilliam, M.; Lorenz, B.; Wenner, A.; Thorp, R. Occurrence and distribution of *Ascosphaera apis* in North America: Chalkbrood in feral honey bee colonies that had been in isolation on Santa Cruz Island, California for over 110 years. *Apidologie* **1997**, *28*, 329–338.
76. Alizon, S.; de Roode, J.C.; Michalakis, Y. Multiple infections and the evolution of virulence. *Ecol. Lett.* **2013**, *16*, 556–567.
77. Alizon, S.; Hurford, A.; Mideo, N.; Van Baalen, M. Virulence evolution and the trade-off hypothesis: History, current state of affairs and the future. *J. Evol. Biol.* **2009**, *22*, 245–259.
78. Klinger, E.G.; Vojvodic, S.; DeGrandi-Hoffman, G.; Welker, D.L.; James, R.R. Mixed infections reveal virulence differences between host-specific bee pathogens. *J. Invertebr. Pathol.* **2015**, *129*, 28–35.
79. Brown, S.P.; Hochberg, M.E.; Grenfell, B.T. Does multiple infection select for raised virulence? *Trends Microbiol.* **2002**, *10*, 401–405.
80. Buckling, A.; Brockhurst, M. Kin selection and the evolution of virulence. *Heredity* **2008**, *100*, 484–488.
81. Gilliam, M. Infectivity and survival of the chalkbrood pathogen, *Ascosphaera apis*, in colonies of honey bees, Apis mellifera. *Apidologie* **1986**, *17*, 93–100.

Inhibitory effect of selected botanical compounds on the honey bee fungal pathogen *Ascosphaera apis*

Petr Mraz[1], Andrea Bohata[1], Irena Hostickova[1], Marek Kopecky[2], Martin Zabka[3], Marian Hybl[4], Vladislav Curn[1]

[1]Department of Plant Production

[2]Department of Agroecosystems

University of South Bohemia in Ceske Budejovice

Studentska 1668, 370 05 Ceske Budejovice

[3]Crop Research institute

Drnovska, 507, 161 06 Praha

[4]Department of Zoology, Fisheries, Hydrobiology and Apidology

Mendel University in Brno

Zemedelska 1, 613 00 Brno

CZECH REPUBLIC

mrazpe01@jcu.cz

Abstract: *Ascosphaera apis* is a heterothallic fungus causing widespread honey bee disease called chalkbrood. In many countries, beekeepers use fungicides to control this pathogen. However, this approach results in residues in bee products as well as in resistance which are serious problems. For these reasons, in European Union countries is not allowed to use nor fungicides, neither antibiotic to control bee diseases. Therefore, there is an increasing need to find environmentally friendly methods to control honey bee pathogens. One of the most promising approaches seems to be using natural botanical compounds with antifungal effects. In this paper, 2 essential oils and 2 main components were tested for *A. apis* inhibition in *in vitro* conditions. Local strain of *A. apis* was cultivated on Sabouraud dextrose agar (SDA) with different concentrations of tested botanical compounds. Cultivation was carried out at 30 °C for 21 days. The greatest inhibitory effect reached thymol (MIC 500 µg/ml). Very promising seems to be also clove bud oil (MIC 2500 µg/ml) and eugenol (MIC 2500 µg/ml). Cedarwood oil did not stop the growth of *A. apis* even in the highest concentration (MIC >5000 µg/ml). This experiment confirmed that these plant substances are efficient as an antimicrobial agent against chalkbrood disease. Despite problems with unstable botanical compounds composition in the same plant species, application of essential oils and their main components could be gentle and safe way how to control a lot of bee diseases in the future.

Key Words: Essential oils, chalkbrood, cultivation media, cultivation, antifungal effect

INTRODUCTION

More than three quarters of agricultural crops are dependent on pollination and approximately one third of food production is influenced by pollinators (Klein et al. 2007). Therefore, reduction in abundance of insects and their extinction draw the attention to many scientists because it poses a significant threat. The same, if not worse situation affects also wild plants (Biesmeier et al. 2006). One of the most important pollinators is honey bee (*Apis mellifera*) which is currently affected by many diseases more frequently than before. It is the result of exposure of bees to pesticides applied to the fields and antibiotics to bee hives (Sandrock et al. 2014), decline of biodiversity and pollen mono diet, transfer of bee colonies for long distances, inadequate beekeeping practice and also anthropogenic modifications in habitats and climate changes (Biesmeier et al. 2006). One of the very common and worldwide spread diseases is chalkbrood. Its incidence is on the rise and may cause significant economic losses, especially in cold and damp weather conditions (Aronstein and Murray 2010). There are some reports from East Asia where beekeepers deal with this disease very often (Chantawannakul et al. 2005).

Chalkbrood is caused by heterothallic fungus *Ascosphaera apis* (Maassen ex Claussen) (Spiltoir 1955) which is closely specialized to honey bees and it can infect only bee larvae. Spores of this fungus can be consumed with food by honey bee larvae. In the suitable conditions in a gut, spores are activated by higher concentration of carbon dioxide and the mycelium starts grow. At the beginning of infection, the larvae cease food consumption and get swollen. During the time, mycelium grows throughout the larvae and causes its dead. After some time, the larvae are going to shrink and solidify and also form so-called chalkbrood mummy. The color of cadavers can be white, black/grey or mottled depend on the presence of ascospores being created on the surface. These ascospores, which can be produced only sexually, are placed into resistant cyst and stay viable for many years. The mummies resemble chalk, from that the name of the disease originated (Aronstein and Murray 2010).

In many countries, beekeepers still use fungicides to treat chalkbrood disease. It can, however, leave residues in bee products and also causes problem with pathogen resistance (Chaimanee et al. 2017). Therefore, in European Union countries, are these substances banned and there is no registered therapeutic agent against chalkbrood. A lot of chemotherapeutic compounds have been investigated as potential substances to treat chalkbrood. Although many of them have antifungal effect, they are not efficient to spores and, in addition, they have negative effect to bee vitality and longevity (Aronstein and Murray 2010). Moreover, there is also a worldwide increase prevalence of other fungal pathogen species on fields and warehouses which are associated with higher fungicide consumption (Zabka et al. 2014). Farmers and even consumers are exposed to long-term, low-dose unnatural substances due to fungicides residues in food supply and groundwater as well as many non-target organisms, especially in developing countries. These negative substances are linked to immune suppression, hormone disruption and cancer. Thus, there is an increasing effort to use alternative, more environmentally friendly methods. Recently, one of the most promising way how to control chalkbrood seems to be using of plant essential oils (EOs) or their main components (MCs) with fungicide effect (Gabriel et al. 2018). Their next substantial advantage is that they are allowed in human food chain included honey production because of GRAS status (Chantawannakul et al. 2005, Li et al. 2019).

A various plants had been used in medicine long time before microbes were discovered. Our ancestors empirically observed the healing potential of some plant species which is currently known as the antimicrobial effect. This effect is mainly caused by essential oils which are volatile substances of plants containing a various organic bioactive compounds. They are known for its very sharp aroma and taste and have a protective function in plants (Kuzyšinová et al. 2016). Even now, these plant substances are abundantly used in preventing and treating various digestive and respiratory diseases in the form of tea, spices and traditional remedies. They are still efficient because there is no resistance against these compounds. In recent time, there is an increasing effort to utilize these plant compounds because they are easily degradable and environmentally friendly and have minimum or no side effects (Li et al. 2019). In this paper, we demonstrated antifungal effect of some botanical compounds against the local strain of *A. apis,* which have a potential to be used as agents against honey bee pathogens.

MATERIAL AND METHODS

Fungal isolates and essential oils

Ascosphaera apis isolates were obtained from mummified bee brood collected on apiary in South Bohemia. The opposite mating types were separated on the PDA (Potato dextrose agar, Himedia) with addition of chloramphenicol by sub culturing until pure culture were established and stored as the maternal cultures on PDA at 30 °C. For antifungal effect, the male mating type was chosen due to its faster growth. For fungicide activity assay, two EOs (clove bud oil, cedarwood oil) and two MCs (thymol, eugenol) were chosen based on their antimicrobial activity. All of these substances were obtained from Sigma Aldrich and stored in dry, dark conditions at 22 °C. The DMSO (dimethyl sulfoxide, Himedia) served as a solvent. Four different concentrations of each tested substances were prepared by diluting 25, 50, 250 and 500 µl of EOs or MCs in 500 µl of DMSO for 100 ml media.

Testing of EOs and MCs

For cultivation tests, SDA (Sabouraud dextrose agar, Himedia) medium was used due to its suitability for *A. apis* growth and also for the medium uniformity. A volumes of 100 ml SDA media were autoclaved at 121 °C for 30 min in Erlenmeyer flask for each variant. Diluted substances of EO/MC were added to the autoclaved SDA cooled at 45 °C aseptically to ensure final concentrations (250, 500, 2500, 5000 µg/ml) and dispersed by circular movements. An amount of 20 ml were poured into Petri dishes (90 x 15 mm). After media solidification, the 8 mm cork bores of mycelium were cut from the edge of 7 days old culture and placed in the middle of each Petri dish. Five repetitions were prepared for each concentration of tested substances. *A. apis* was cultivated under the dark condition at 30 °C for 21 days. The growth of mycelium was measured by two perpendicular diameters daily for the first 10 days, then the 15th day and the 21st day. The inhibitions effect was calculated. Data from the 10th day of cultivation were statistically analysed with ANOVA (STATISTICA 12, StatSoft Inc.) and the mean values were compared using the Tukey HSD test ($p < 0.05$).

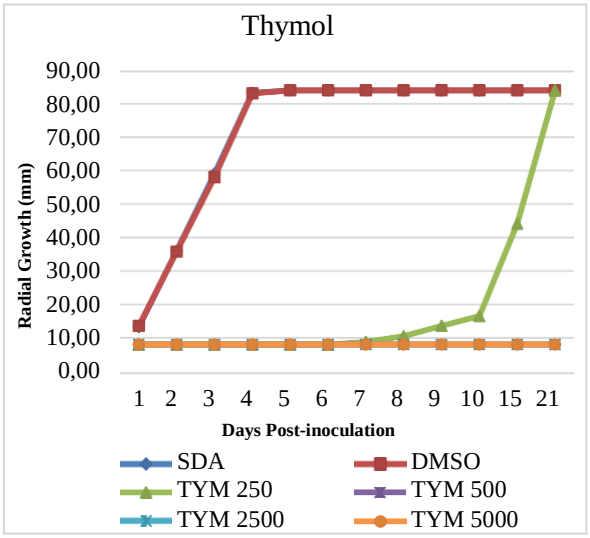
RESULTS AND DISCUSSION

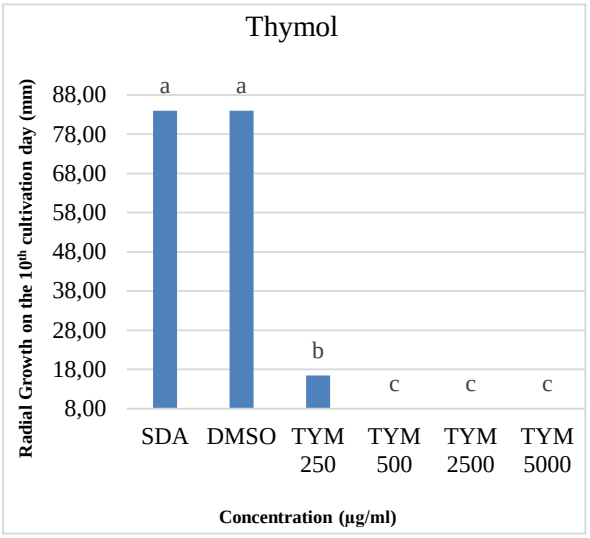
An antifungal effect of four plant substances was tested against *A. apis*. This fungus grew very well on SDA medium as well as on the SDA controls including the DMSO. All the tested substances showed an antifungal effect (Figure 1). In each case, the higher concentration of tested botanical compounds caused higher growth inhibition. Thymol ($F_{(5,54)} = 32315.7$, $p < 0.05$) has been detected as the most effective substances with the MIC < 500 µg/ml after 10 days of cultivation. However, during the first 7 days, there was no *A. apis* growth even at the lowest concentration (250 µg/ml). Calderone et al. (1994) determined its MIC as 1000 µg/ml after 7 days of cultivation. This variability may indicate different sensitivity of individual *A. apis* strains or different substances composition. Thymol is considered as a broad range inhibitor because it can suppress growth many fungal species as *Alternaria alternate*, *Stachybotrys chartarum*, *Cladosporium cladosporioides* and especially significant for beekeeping *Aspergilus niger* causing Stonebrood disease (Zabka et al. 2014) as well as *Paenibacillus. larvae* causing American foulbrood (Fuselli et al. 2006). This substance was tested against many different bacteria and yeast with very promising results (Wiese et al. 2018). Thyme, with its main component thymol, had very low MIC (300 µg/ml) also against the human pathogen as *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* (Hammer et al. 1999).

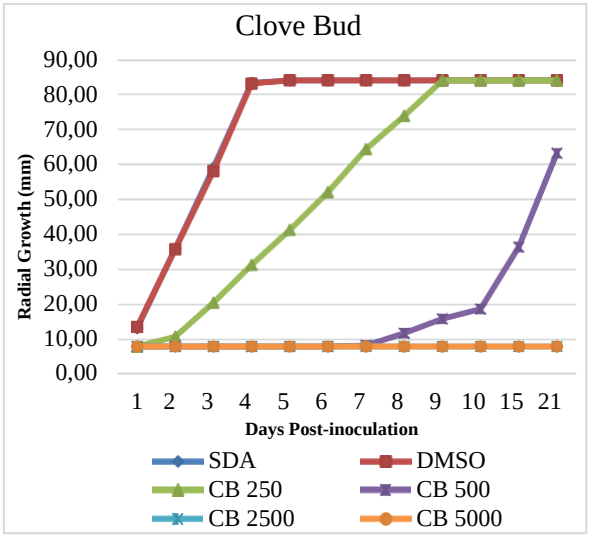
Great antifungal effect had also clove bud oil ($F_{(5,54)} = 47413.8$, $p < 0.05$) with MIC < 2500 µg/ml measured after 10 days of cultivation. According to Chaimanee et al. (2017), clove bud EO had MIC very low (32 µg/ml) after 3 days of cultivation and as an efficient oil is also considered by Ansari et al. (2016) with MIC 400 µg/ml and by Calderone et al. (1994) with MIC 1000 µg/ml, both measured after 7 days of cultivation. In our study, the lowest concentration was relatively harmless and mycelium started to grow even on the second day of cultivation. However, the clove bud concentration of 500 µg/ml inhibited *A. apis* growth for the first 7 days (Figure 1). Clove bud oil is efficient also against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* (MIC 1200 µg/ml) representing a group of common human pathogens (Hammer et al. 1999). Especially clove bud oil main component, eugenol, is known for its significant antifungal effect. In this study, eugenol inhibited 100% *A. apis* growth at 2500 µg/ml after 10 days of cultivation ($F_{(5,54)} = 11342$, $p < 0.05$). Its particular efficiency was proved also by Larrán et al. (2001) who tested eugenol as a part of basil oil against *A. apis* and also by Gende et al. (2008) who tested eugenol as a main part of *Cinnamomum zeylanicum* against *P. larvae*.

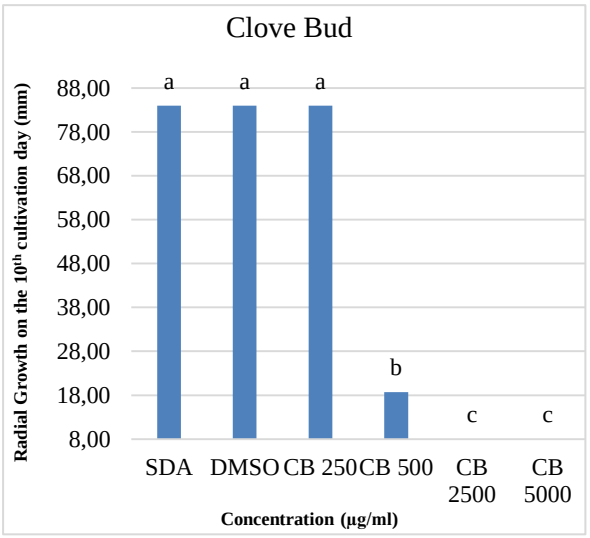
Despite cedarwood essential oil ($F_{(5,54)} = 3876.25$, $p < 0.05$) showed antimicrobial activity (Figure 1), it did not completely inhibit *A. apis* growth in any tested concentrations (MIC >5000 µg/ml). For this reason, its use can be suitable only assuming its synergic effect with other medical substances to treat chalkbrood disease. In cedarwood oil were recorded considerable diversity in its compositions, therefore the efficacy can differ. The same effect occurs in many plant oils (Paoli et al. 2011).

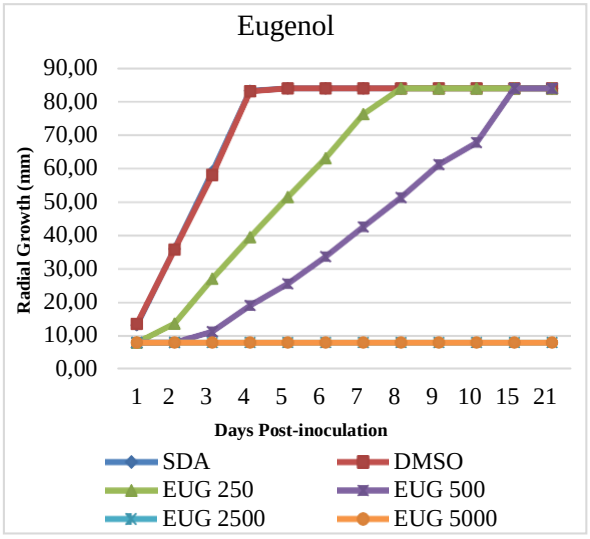
Figure 1 Inhibition effect of botanical compounds on the A. apis pathogen during 21 days, differences among the concentrations on the 10th day of fungus cultivation show small letters (HSD Tukey test)

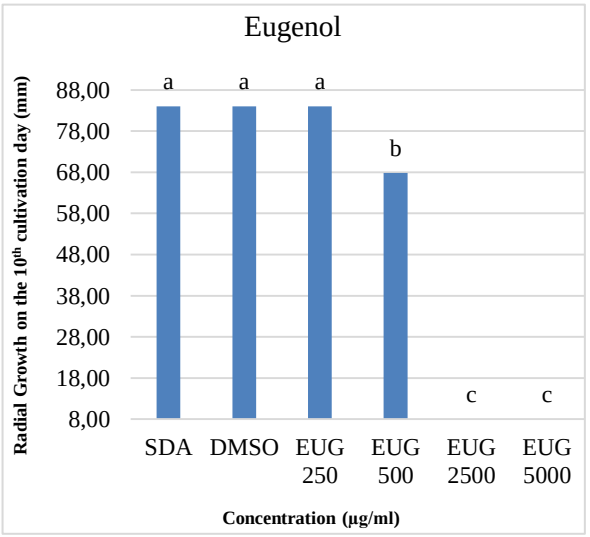


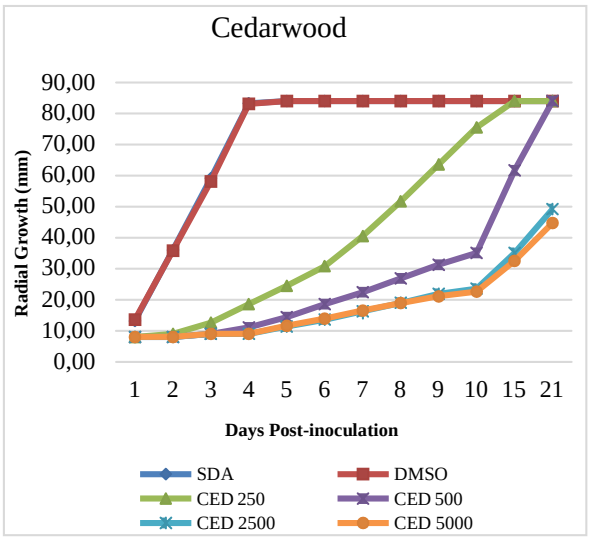


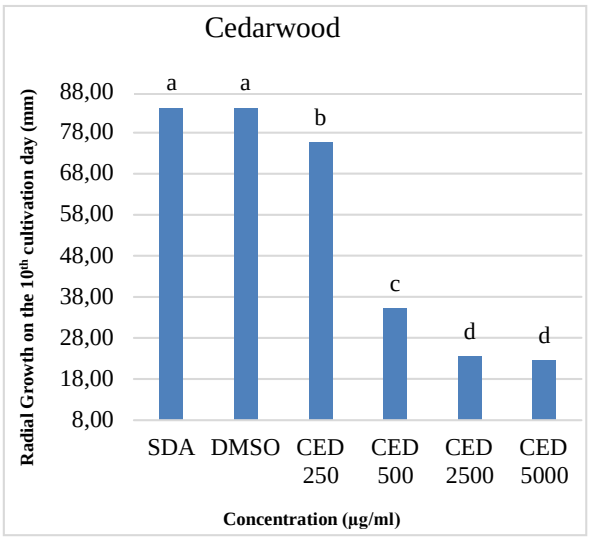












CONCLUSIONS

Plants contain a diverse composition of essential oils and their main components and a lot of them have an antimicrobial effect. By these botanical compounds, plants can protect themselves against many pests and pathogens. Therefore, essential oils are used for plant protection in agriculture and it is considered as an environmentally friendly way with a minimum risk of acquisition of resistance. Moreover, there are no residues in agriculture products after their use because essential oils are easily degradable. In addition, they are convenient also for organic farming. For these reasons, botanical compounds have a large potential and represent a suitable alternative to prevent or treat different kind of bee diseases. Their antifungal effect was confirmed also by this study. There were tested four plant substances and especially thymol and clove bud essential oil had significant antimicrobial effect against *A. apis*. It is very likely, that some substances can cause a synergistic effect, which would increase antimicrobial activity and decrease costs for its practical use. However, the next studies are needed to confirm bee tolerance and also to find suitable methods for its application in bee hives.

ACKNOWLEDGEMENTS

This study was supported by grants MZe ČR – RO0418, NAZV QK1910356 and GAJU 027/2019/Z.

REFERENCES

Ansari, M.J. et al. 2016. *In vitro* evaluation of the effects of some plant essential oils on *Paenibacillus larvae*, the causative agent of American foulbrood. Biotechnology & Biotechnological Equipment, 30(1): 49–55.

Aronstein, K.A., Murray, K.D. 2010. Chalkbrood disease in honey bees. Journal of Invertebrate Pathology, 103: 20–29.

Biesmeijer, J.C. et al. 2006. Parallel declines in pollinators and insect pollinated plants in Britain and the Netherlands. Science, 313: 351–354.

Calderone, N.W. et al. 1994. An *in vitro* evaluation of botanical compounds for the Honeybee pathogens *Bacillus larvae* and *Ascosphaera apis*, and the secondary invader *B. alvei*. Journal of Essential Oil Research, 6: 279–287.

Chaimanee, V. et al. 2017. Antimicrobial activity of plant extracts against the honeybee pathogens, *Paenibacillus larvae* and *Ascosphaera apis* and their topical toxicity to *Apis mellifera* adults. Journal of Applied Microbiology, 123(5): 1160–1167.

Chantawannakul, P. et al. 2005. Inhibitory effects of some medicinal plant extracts on the growth of *Ascosphaera apis*. Acta Horticulturae, 678: 183–189.

Fuselli, S.R. et al. 2006. Antimicrobial activity of some Argentinian wild plant essential oils against *Paenibacillus larvae larvae*, causal agent of American foulbrood (AFB). Journal of Apicultural Research, 45(1): 2–7.

Gabriel, K.T. et al. 2018. Antimicrobial activity of essential oils against the fungal pathogen *Ascosphaera apis* and *Pseudogymnoascus destructans*. Mycopathologia, 183(6): 921–934.

Gende, L.B. et al. 2008. Antimicrobial activity of cinnamon essential oil and its main components against *Paenibacillus larvae* from Argentine. Bulletin of Insectology, 61(1): 1–4.

Hammer, K.A. et al. 1999. Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology, 86: 985–990.

Klein, A.M. et al. 2007. Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society B: Biological Sciences, 274: 303–313.

Kuzyšinová, K. et al. 2016. The use of probiotics, essential oils and fatty acids in the control of American foulbrood and other bee diseases. Journal of Apiculture Research, 55(5): 386–395.

Larrán, S. et al. 2001. *In vitro* fungistatic effect of essential oils against *Ascosphaera apis*. Journal of Essential Oil Research, 13: 122–124.

Li, X. et al. 2019. Review, Traditional uses, chemical constituents and biological activities of plants from the genus *thymus*. Chemistry & Biodiversity, 10.1002/cbdv.201900254.

Paoli, M. et al. 2011. Chemical variability of the wood essential oil of *Cedrus atlantica manetti* from Corsica. Chemistry & Biodiversity, 8(2): 344–351.

Sandrock, Ch. et al. 2014. Impact of chronic neonicotinoid exposure on honeybee colony performance and queen supersedure. PLOS ONE, 9(8): e103592.

Spiltoir, C.F. et al. 1955. Life cycle of *Ascosphaera apis* (*Pericystis apis*). American Journal of Botany, 42(6): 501–508.

Wiese, N. et al. 2018. The terpenes of leaves, pollen, and nectar of thyme (*Thymus vulgaris*) inhibit growth of bee disease-associated microbes. Scientific Reports, 8: 14634.

Zabka, M. et al. 2014. Antifungal activity and chemical composition of twenty essential oils against significant indoor and outdoor toxigenic and aeroallergenic fungi. Chemosphere, 112: 443–448.

5.7

Antifungal activity of selected botanical compounds on *Ascosphaera apis*

Petr Mráz[a,] *, Martin Žabka[b], Marian Hýbl[a], Marek Kopecký[a], Andrea Bohatá[a], Aleš Tomčala[c], Vladislav Čurn[a]

[a] University of South Bohemia in Ceske Budejovice
Studentska 1668, 370 05 Ceske Budejovice
CZECH REPUBLIC

[b] Crop Research institute
Drnovska, 507, 161 06 Praha
CZECH REPUBLIC

[c] Institute of Aquaculture and Protection of Waters
South Bohemian Research Centre of Aquaculture and Biodiversity of Hydrocenoses
University of South Bohemia in Ceske Budejovice
Husova tr. 458/102, 370 05 Ceske Budejovice
CZECH REPUBLIC

* Corresponding authors at: University of South Bohemia in Ceske Budejovice
Studentska 1668, 370 05 Ceske Budejovice, CZECH REPUBLIC
email: mrazpe01@jcu.cz

Highlights

- 4 essential oils had significant antifungal effect
- Botanical substances inside clove bud and cinnamon oils cause synergistic effect
- Cedarwood oil inhibit the fungal growth even at low concentrations
- Results suggest a potential these oils for use as an alternative to the fungicides

Abstract

Ascosphaera apis is a worldwide spread fungal pathogen invading honey bee brood and causing chalkbrood disease. In many countries, its control with fungicides is banned due to residue contamination of bee products and health risk. Since then, there is no alternative treatment. In this study, an antifungal activity of 10 essential oils or botanical components has been investigated with the potential to participate in alternative methods of controlling the pathogen. Agar dilution method was carried out showing considerable differences between

their antifungal activity with the best results of thyme oil (MIC_{50}= 57.7 ppm) followed by cedarwood oil (MIC_{50}= 64.9 ppm), clove bud oil (MIC_{50}= 114.2 ppm) and cinnamon oil (MIC_{50}= 136.6 ppm). The composition and quantity of main components of above mentioned oils were determined by GC-MS/MS. Thymol is responsible for the antifungal activity of thyme oil, whereas, in clove bud and cinnamon oil, the main component is eugenol. Cedarwood oils contain primarily gama and alpha cendrene. Since the analyzed compounds have a broad range of antimicrobial activity and a possible synergistic effect could increase their efficacy, results suggest they might be used to control several honey bee pathogens at once.

Keywords: Agar dilution method; chromatography; essential oils; fungal pathogen; honey bee; minimum inhibitory concentration

1. Introduction

Managed western honey bees (*Apis mellifera L.*) are kept for their honey production as well as other bee products, especially beeswax, propolis, and fermented pollen. People have used these products all around the world, which makes it important sources (Cianciosi et al., 2018; Ruoff and Bogdanov, 2004). However, the greatest importance of bees lies in the pollination service, because *A. mellifera* belongs to one of the main pollinators worldwide.

Honey bees are stressed by many factors, including malnutrition (Dolezal et al., 2019; Smart et al., 2019), long-distance transport, inadequate beekeeping practice (Biesmeijer et al., 2006), and especially, application of fungicides, miticides, and antibiotics to the beehives as well as pesticides to the fields which often act synergistically (Iwasa et al., 2004; Schmuck et al., 2003; Thompson et al., 2014). These factors make bees more susceptible to parasites and pathogens, which honey bees would be able to fend off in non-stressing conditions. Therefore, honey bees suffer from parasites and pathogens more often these days and there is an

increasing concern about honey bee colony collapses (Genersch, 2010; Goulson et al., 2015; Kulhanek et al., 2017) as well as other insect pollinators (Hallmann et al., 2017; Potts et al., 2010).

Periodical honey bee colony collapses worldwide in recent time lead to insufficient production of honey bee products (Biesmeijer et al., 2006). That increases their demand and thus occurrence of counterfeit (falsified) products (Sahlan et al., 2019). Apart from that, a big amount of cheaper honey from distant countries has been imported to the EU and the USA. In some cases, it can contain residues of pesticides and antibiotics (Bargańska et al., 2011; Ruoff and Bogdanov, 2004). Beeswax has also been contaminated by pesticides, both in Europe (Morales et al., 2020; Vázquez et al., 2015) and USA (Frazier et al., 2008) as well as in Africa (Llorens-Picher et al., 2017), which degrades it and the need of high-quality beeswax is getting bigger. Furthermore, beeswax, too, is falsified with paraffin because of its shortage (Svečnjak et al., 2019). Another bee-related issue is the so-called pollination crisis. Pollination dependent crops are cultivated in a larger area every year and despite a great contribution of bumblebees, wild bees, and solitary bees, there are not sufficient numbers of pollinators to ensure stable yields, which leads to great economic losses (Aizen et al., 2009; Gallai et al., 2009). Neither in wild landscape is the situation better because the lack of pollinators leads to the decline of plant biodiversity (Hallmann et al., 2017).

The very common and widespread bee brood disease is a chalkbrood. It is caused by the pathogen *Ascosphaera apis* (Maassen ex Claussen) (Spiltoir, 1955). This heterothallic fungus can invade only bee brood. Once the ascospores are ingested with contaminated food, they are activated by CO_2 exposure and start to germinate in the gut. Later on, mycelium breaks the peritrophic membrane and grows throughout the larvae. At the beginning of infection, the larvae cease food consumption, get swollen and covered by fluffy mold. In the end, the larvae shrink and resemble white or gray/black chalk, depending on the presence of

ascospores. They are called chalkbrood mummies and represent a typical symptom of the disease (Aronstein and Murray, 2010). Chalkbrood occurs especially in damp and cold weather conditions or in weak bee colonies where workers are unable to maintain a constant temperature at 35°C (Evison, 2015) because *A. apis* prefer rather lower temperatures around 30°C (Mráz et al., 2021). Therefore, disease occurrence in beehives is promoted by exposing bee larvae to cold-stress (Vojvodic et al., 2011).

Chalkbrood are ordinarily treated chemically by fungicides. Generally, the application of pesticides helps prevent economic losses, including higher crop quality, protection of crop losses and vector-borne disease control, therefore, they bring considerable benefits (Aktar et al., 2009). On the other hand, fungicides leave residues and can cause pathogen resistance (Evans, 2003; Rinkevich, 2020; van den Heever et al., 2014). Furthermore, long-term, low-dose exposure of pesticides has a negative effect even for people because it can cause hormone disruption, suppressed immune function, reproductive abnormalities, and cancer. Pesticides and their residues can also be leached into the groundwater and contaminate drinking water, which is a serious problem (Aktar et al., 2009). Pesticides negatively affect non-target organisms, e.g. those in bee gut microbiota, which leads to a disruption of bee immunity (Zhu et al., 2020). Many studies have investigated the side effects of these pesticides warning against negative influence related to honey bee learning, memory, navigation, foraging activity or immune response (Cullen et al., 2019; Liao et al., 2019; Tison et al., 2019). For these reasons, the use of fungicides in beehives is prohibited in many countries, including the EU. Therefore, there is a great interest in the investigation of alternative and efficient disease-controlling substances (Ansari et al., 2017).

Several approaches or alternatives seem to be promising. One of them is using essential oils (EOs) containing biologically active compounds, especially phenols, terpenes, and terpenoids (Zabka and Pavela, 2013). They are known for the antimicrobial effects (Rios

and Recio, 2005) that mankind has been using since the Middle Ages (Bakkali et al., 2008). That is one of the main reasons they are considered safe in the human food chain and also environmentally-friendly (Chantawannakul et al., 2003). As a natural product, its application does not leave residues, because essential oils are completely degradable. Therefore, essential oils are convenient also for organic farming. Furthermore, thanks to many biologically active substances composition, it is very difficult to involve resistance for the pathogens (Milano and Donnarumma, 2017). Essential oils could represent an alternative to the synthetics fungicides, and thus reduce the environmental pollution (Du Plooy et al., 2009).

Originally, Essential oils are formed in various aromatic plants as secondary metabolites and play an important role in their protection as antimicrobial agents (Bakkali et al., 2008). Besides, they may attract pollinators to ensure plant reproduction (Stevenson, 2020). For commercial use, essential oils are the product of distilled, pressed, or extracted all part of raw plant organs including buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark, resulting in a concentrated solution of volatile organic compounds characterized by a strong odor (Ferrentino et al., 2020).

Several studies have been carried out investigating the inhibitory effect of essential oils on honey bee pathogens and parasites, namely *A. apis* (Ansari et al., 2017; Calderone et al., 1994; Chaimanee et al., 2017; Chantawannakul et al., 2003; Gabriel et al., 2018), *P. larvae* (Calderone et al., 1994; Chaimanee et al., 2017; Damiani et al., 2014), *V. destructor* (Ariana et al., 2002; Conti et al., 2020; Damiani et al., 2009), and *N. ceranae* (Borges et al., 2020; Bravo et al., 2017) with promising results. Apart from the main components (MCs), essential oils contain other numerous molecules in small quantities. These can play a role in cell penetration, lipophilic or hydrophilic attraction and fixation on cell membranes or cellular distribution. Thus, they can act synergically and increase the antimicrobial effect (Bakkali et al., 2008). What more, phenols, which commonly occurred in essential oils can reduce the

mortality of honey bees intoxicated by pesticides (Hýbl et al., 2021). Unfortunately, essential oils' biologically active substances proportion varies depending on a part of the plant, climate, soil composition, plant age, vegetative cycle stage, and genetic traits. This fluctuating composition of substances complicates the use of essential oils (Angioni et al., 2006; Masotti et al., 2003; Tock et al., 2020).

This study aims to verify the inhibition effect of selected essential plant oils and their main components on the widespread honeybee pathogen *Ascosphaera apis.* First, the minimum inhibition concentrations of botanical substances were evaluated, and second, the chemical compositions of the most effective oils were determined.

2. Material and methods

2.1.Fungal culture, cultivation media and essential oils

The *Ascosphaera apis* strain was isolated from mummified bee broods from the South Bohemia region. The species was identified by molecular methods, specifically sequencing of ITS region and comparison with the BLAST (The National Centre for Biotechnology Information; NCBI). The male mating type was chosen for the inhibition tests due to its faster growth (Mráz et al., 2021). The isolate was grown on SDA (Sabouraud dextrose agar, Himedia) at 30°C. Essential oils and main components were obtained from Sigma-Aldrich, Prague, Czech Republic.

2.2.Agar dilution method

The agar dilution method was carried out according to Zabka et al., (2009) with small modifications. Briefly, an amount of 25, 50, 250 and 500 µl of EOs or MCs were dissolved in 500 µl DMSO and mixed with a 100ml SDA medium tempered at 45°C. The emulsions were dispersed by circular movements and 20 ml of the media was poured into Petri dishes (90x15 mm). The final concentrations were 250, 500, 2500 and 5000 ppm. The medium SDA with

5 000 ppm DMSO was used as a negative control. Untreated and uninoculated SDA was used as a contamination control. Once the media solidified, an 8 mm diameter plug was removed from the edge of 7 days old culture of *A. apis* and placed in the middle of each Petri dish. Each experiment was conducted in five repetitions. Plates were incubated at 30 °C in dark conditions until the cultures reached the edge of the Petri dish or ceased the growth. The radial growth was measured by two perpendicular diameters daily. The percent inhibition of the radial growth of the target fungi was calculated according to the following formula. Percent inhibition = (DC − DT)/DC × 100, where DC is the colony diameter of the control sets and DT is the colony diameter of the treated sets. The second sets of experiments were done with a narrower range of concentration based on the results of the first experiment. The concentrations were as follows (ppm): Cedarwood - 5, 10, 25, 50, 100, 200, 250, 400; thyme - 50, 100, 200; clove bud, cinnamon, eugenol, wintergreen - 100, 200, 400; methyl eugenol 100, 200, 500; peppermint - 250, 500, 1000, Linalool - 600, 1200, 2000; Lavandin 600, 1200, 2500. From the obtained data, a MIC_{50} was calculated.

2.3.Determination of main components by GC-MS/MS

Samples of essential oils were analysed diluted 1:10000 in hexane by GC MS/MS system consisting of TriPlus autosampler, Trace GC Ultra gas chromatograph equipped with a TG-5MS fused silica capillary column, 30 m × 0.25 mm × 0.25 μm, and coupled to a mass spectrometer TSQ Quantum XLS all from Thermo Fischer Scientific, USA. Helium was used as a carrier gas at 1.0 ml/min. 1 μl of the sample was injected to SSL injector in the splitless mode set at 280 °C. The oven temperature was programmed as follows: start at 40 °C and held for 5 min, then increased to 150 °C at a rate of 3 °C/min and held for 0.5 min, then increased to 250 °C at a rate of 10 °C/min, then increased to 290 °C at a rate of 25 °C and finally maintained at 290 °C for 10 min. The temperature of the transfer line was held at 250 °C, and the ion source was operating at 200 °C. TIC mode was performed on Q1 at 70 eV of

ionization energy and mass range 50 – 450 m/z. To exclude congestion of detector the scanning was performed after 6 min of injection. The data was processed in Thermo Xcalibur 3.0.63 (Thermo Fisher). Components identification was made based on comparison with the NIST Mass Spectral Search Program library v 2.0f (Thermno Fisher). The quantification was achieved based on Q3 SIM mode focused on fragmentation ions of desired compounds and also via external calibration curve. The Thujone (Sigma Aldrich) was used as an internal and also external standard.

3. Results

All the tested botanical substances inhibit the growth of *A. apis* at various concentrations (Tab 1). The greatest inhibitory effect was caused by thyme oil (MIC_{50} = 57.7 ppm). Very high efficiency had also cedarwood (MIC_{50} = 64.9 ppm), clove bud (MIC_{50} = 114.2 ppm) and cinnamon oil (MIC_{50} = 136.6 ppm). From the MCs, the highest inhibitory effect had methyl eugenol (MIC_{50} = 146.5 ppm) and eugenol (MIC_{50} = 151.1 ppm). Moderate efficiency had wintergreen and low efficiency had peppermint oil, linalool and lavandin.

Table 1. Inhibitory effect of EOs/selected natural compounds on *A. apis* at 2500 ppm and MIC_{50}

Tested substance	Inhibition (%)	S.D. (%)	MIC_{50}[a] (ppm)	CI[99b]	Chi square[c]
Thyme	100	±0.00	57.7	50.22-64.53	0.009
Cedarwood	100	±0.00	64.9	57.69-73.04	0.465
Clove bud	100	±0.00	114.2	78.29-140.47	0.246
Cinnamon	100	±0.00	136.6	117.77-154.26	0.192
Methyl eugenol	100	±0.00	146.5	114.96-176.52	0.459

Eugenol	100	±0.00	151.1	138.12-163.77	0.269
Wintergreen	100	±0.00	209.9	163.95-257.66	0.096
Peppermint	100	±0.00	556.7	478.48-656.42	0.003
Linalool	100	±0.00	806.0	709.56-917.11	0.193
Lavandin	87.5	±2.99	1062.7	924.92-1233.67	0.380

[a]Minimum inhibitory concentration of compound that resulted in a 50% inhibition

[b]99% confidence intervals

[c]Chi-square value, significant at $P < 0.01$ level

The chemical composition of the tested essential oils is given in table 2. The main component of clove bud oil was eugenol (62.6%) and acetyleugenol (31.7%). Likewise cinnamon contained eugenol (88.6%) as a main component. Thyme oil contained mainly thymol (69.4%) and linalool (10%). Cedarwood contained more main substances in lower concentrations, from which the most frequent was gama cendrene (31.9%) and alpha cendrene (31.9%) followed by beta cendrene (14.3%) and cuparene (10%).

Table 2. Chemical composition of the tested essential oils

Component	RT [min]	Normalized area [%]			
		Cedarwood	Clove bud	Cinnamon	Thyme
Linalool	21,13	0,15	0,33	6,63	10,03
Camphor	23,17	0,06	0,03	0,07	3,52
Borneol	24,32	0,03	0,04	0,33	6,74
Estragole	29,95	2,44	0,00	0,00	0,00
Thymol	30,67	0,00	0,14	0,00	69,41
Eugenol	33,11	1,53	62,59	88,60	4,95

Anisketone	34,18	0,41	0,00	0,00	0,00
gama_cedrene	35,65	31,88	4,39	4,37	5,35
alfa_cedrene	35,65	31,85	0,00	0,00	0,00
beta_cedrene	35,97	14,27	0,00	0,00	0,00
beta-Vetirenene	37,70	7,31	0,80	0,00	0,00
Cuparene	39,42	10,07	0,00	0,00	0,00
Acetyleugenol	40,13	0,00	31,67	0,00	0,00

4. Discussion

This study deals with the antifungal effect of several essential oils and botanical compounds on the honey bee brood pathogen *Ascosphaera apis*. All the tested botanical substances inhibit the growth of *A. apis* at various concentrations (Tab. 1) due to its different composition (Tab. 2). The most effective oils were thyme, cedarwood, clove bud, and cinnamon. Therefore, GC-MS/MS analyses were performed to reveal their main components. The greatest inhibitory effect was caused by thyme oil (MIC_{50} = 57.7 ppm). The main component of thyme oil is thymol (69.4%), which has a high antimicrobial activity on the fungal pathogens (Boudegga et al., 2010; Calderone et al., 1994; Kloucek et al., 2012; Zabka et al., 2014) and also on other honey bee pathogens, specifically *Melissococcus plutonius* (Wiese et al., 2018), *Paenibacillus larvae* (Alippi et al., 1996; Flesar et al., 2010; Fuselli et al., 2009), *Nosema ceranae* (Borges et al., 2020; Costa et al., 2010; Maistrello et al., 2008) and the parasite *Varroa destructor* (Ariana et al., 2002; Damiani et al., 2009). Even a low concentration of thymol naturally occurred in nectar and pollen have an antimicrobial effect (Stevenson, 2020; Wiese et al., 2018). Furthermore, thyme oil and its main component thymol have a low toxicity on adult honey bees (Ariana et al., 2002) as well as honey bee larvae (Charpentier et al., 2014) and other pollinators (Stevenson, 2020). Therefore, it is considered

safe for honey bees (Gashout et al., 2020; Gregorc et al., 2018) but there are also studies which make its safety controversial (Alayrangues et al., 2016; Chapuy et al., 2019; Colin et al., 2019). Despite that, thymol has already been registered as a varroacide in several products.

Cedarwood oil was the second most effective EO (MIC_{50} = 64.9 ppm) on the fungal pathogen. There is no evidence in literature how the oil influences honey bees or their pathogens. However, it shows high antimicrobial activity on bacteria (Korona-Glowniak et al., 2020; Zrira and Ghanmi, 2016) and has also sporicidal activity (Ramadass and Thiagarajan, 2015). According to our results, cedarwood oil reduced the growth rate at low concentrations for a long period of time. This effect could be used in different EOs mixtures. For that effect, active constituents with a slow rate of release could be responsible. The main components of cedarwood oil are gama and alpha cendrene, which represent 63.7% of total oil composition. Next abundant compounds are beta cendrene (14.3%), cuparene (10%) and beta-vetirene (7.3%). However, its composition often differs significantly (Adams, 1991).

The moderate inhibition effect caused clove bud oil (MIC_{50} = 114.2 ppm), cinnamon oil (MIC_{50} = 136.6 ppm), methyl eugenol (MIC_{50} = 146.5 ppm), and eugenol (MIC_{50} = 151.1 ppm). Especially cinnamon oil is considered to have a great antifungal (Chantawannakul et al., 2003; Gabriel et al., 2018) and antibacterial effect against honey bee pathogens (Calderone et al., 1994; Chaimanee et al., 2017). It is mainly caused by the constituent eugenol (88.6%), which dominated in the oil composition. Its further research is, however, hampered by its high toxicity towards honey bees (Chaimanee et al., 2017), especially at higher concentration (Arismendi et al., 2018).

Better results achieved clove bud oils, particularly from the 500 ppm concentration. A lower MIC_{100} is reported with a shorter monitoring time (Ansari et al., 2017; Chaimanee et al., 2017). Honey bee sensitivity to the oil is lower than to cinnamon oil (Chaimanee et al., 2017), and even lower than thymol, oxalic and formic acid (Ebert et al., 2007) which are commonly used as acaricides in bee hives. In addition, clove bud oil negatively affects *P. larvae* (Calderone et al., 1994; Chaimanee et al., 2017) and *V. destructor* (Li et al., 2017; Maggi et al., 2010). Therefore, clove bud oil has a good potential to take a part in alternative treatments. Its main component is eugenol (62.6%), but it also contains a higher amount of acethyleugenol (31.7%).

Eugenol is a main component of clove bud and cinnamon oil and is responsible for the antimicrobial activity (Maggi et al., 2010) as well as methyl eugenol in many other essential oils, for example *Laurus nobilis* (Nabila et al., 2020) or lemongrass (Lal et al., 2020). Since these MCs had higher values of MIC_{50} (151.1 ppm and 146.5 ppm, respectively) than clove bud and cinnamon oils, it's very likely that a synergistic effect among the oils component occurs. This effect could be used intentionally in practice to increase the antimicrobial activity and to lower the dose and cost of products (Sharma et al., 2020). Several compounds are already known to act synergistically, for example thymol and eugenol (Palmer-Young et al., 2017) or eugenol with some synthetics compounds (Jafri et al., 2020).

Wintergreen and peppermint oils (MIC_{50} = 209.9 ppm and 556.7 ppm) did not show a high antifungal effect as well as linalool and lavandin (MIC_{50} = 806.0 ppm and 1062.7 ppm). However, wintergreen, peppermint, (Imdorf et al., 1999) and lavandin (Ariana et al., 2002; Damiani et al., 2009) oil have been proved to have acaricidal effect against *Varroa destructor*.

5. Conclusion

This research has identified 4 essential oils with high inhibition effect against *A. apis*. Additionally, their main substituents were recognized. Results suggest potential use of these EOs and their MCs as antifungal treatment in beekeeping practice. However, the assessment on the honey bee tolerance needs to be done as well as examination of the essential oil application forms in practical use.

CRediT authorship contribution statement

Petr Mráz: Investigation, Data curation, Methodology, Writing - original draft. **Martin Žabka:** Data curation, Supervision. **Marian Hýbl:** Investigation, Data curation, Writing - original draft. **Marek Kopecký:** Data curation, Methodology. **Andrea Bohatá:** Data curation, Supervision. **Aleš Tomčala:** Data curation, Formal analysis, Funding acquisition. **Vladislav Čurn:** Data curation, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

This research was supported by the Ministry of Education, Youth and Sports of the Czech Republic through the projects: "CENAKVA" [LM2018099] and "CENAKAVA Center Development" [CZ.1.05/2.1.00/19.0380], and Reproductive and Genetic Procedures for Preserving Fish Biodiversity and Aquaculture [CZ.02.1.01/0.0/0.0/16_025/0007370].

References

Adams, R., 1991. Cedar wood oil—Analyses and properties, in: Essential Oils and Waxes. Springer, Berlin, pp. 159–173.

Aizen, M.A., Garibaldi, L.A., Cunningham, S.A., Klein, A.M., 2009. How much does agriculture depend on pollinators? Lessons from long-term trends in crop production. Annals of botany 103, 1579–1588.

Aktar, M.W., Sengupta, D., Chowdhury, A., 2009. Impact of pesticides use in agriculture: their benefits and hazards. Interdisciplinary toxicology 2, 1.

Alayrangues, J., Hotier, L., Massou, I., Bertrand, Y., Armengaud, C., 2016. Prolonged effects of in-hive monoterpenoids on the honey bee Apis mellifera. Ecotoxicology 25, 856–862.
Alippi, A.M., Ringuelet, J.A., Cerimele, E.L., Re, M.S., Henning, C.P., 1996. Antimicrobial activity of some essential oils against Paenibacillus larvae, the causal agent of American foulbrood disease. Journal of herbs, spices & medicinal plants 4, 9–16.
Angioni, A., Barra, A., Coroneo, V., Dessi, S., Cabras, P., 2006. Chemical composition, seasonal variability, and antifungal activity of Lavandula stoechas L. ssp. stoechas essential oils from stem/leaves and flowers. Journal of agricultural and food chemistry 54, 4364–4370.
Ansari, M.J., Al-Ghamdi, A., Usmani, S., Khan, K.A., Alqarni, A.S., Kaur, M., Al-Waili, N., 2017. In vitro evaluation of the effects of some plant essential oils on Ascosphaera apis, the causative agent of Chalkbrood disease. Saudi journal of biological sciences 24, 1001–1006.
Ariana, A., Ebadi, R., Tahmasebi, G., 2002. Laboratory evaluation of some plant essences to control Varroa destructor (Acari: Varroidae). Experimental & applied acarology 27, 319–327.
Arismendi, N., Vargas, M., López, M.D., Barría, Y., Zapata, N., 2018. Promising antimicrobial activity against the honey bee parasite Nosema ceranae by methanolic extracts from Chilean native plants and propolis. Journal of Apicultural Research 57, 522–535.
Aronstein, K.A., Murray, K.D., 2010. Chalkbrood disease in honey bees. Journal of invertebrate pathology 103, S20–S29.
Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils–a review. Food and chemical toxicology 46, 446–475.
Bargańska, Ż., Namieśnik, J., Ślebioda, M., 2011. Determination of antibiotic residues in honey. TrAC Trends in Analytical Chemistry 30, 1035–1041.
Biesmeijer, J.C., Roberts, S.P., Reemer, M., Ohlemüller, R., Edwards, M., Peeters, T., Schaffers, A.P., Potts, S.G., Kleukers, R., Thomas, C.D., 2006. Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. Science 313, 351–354.
Borges, D., Guzman-Novoa, E., Goodwin, P.H., 2020. Control of the microsporidian parasite Nosema ceranae in honey bees (Apis mellifera) using nutraceutical and immuno-stimulatory compounds. Plos one 15, e0227484.
Boudegga, H., Boughalleb, N., Barbouche, N., Ben Hamouda, M.H., Mahjoub, M.E., 2010. In vitro inhibitory actions of some essential oils on Ascosphaera apis, a fungus responsible for honey bee chalkbrood. Journal of apicultural research 49, 236–242.
Bravo, J., Carbonell, V., Sepúlveda, B., Delporte, C., Valdovinos, C., Martín-Hernández, R., Higes, M., 2017. Antifungal activity of the essential oil obtained from Cryptocarya alba against infection in honey bees by Nosema ceranae. Journal of invertebrate pathology 149, 141–147.
Calderone, N.W., Shimanuki, H., Allen-Wardell, G., 1994. An in vitro evaluation of botanical compounds for the control of the honeybee pathogens Bacillus larvae and Ascosphaera apis, and the secondary invader B. alvei. Journal of Essential Oil Research 6, 279–287.
Chaimanee, V., Thongtue, U., Sornmai, N., Songsri, S., Pettis, J.S., 2017. Antimicrobial activity of plant extracts against the honeybee pathogens, Paenibacillus larvae and Ascosphaera apis and their topical toxicity to Apis mellifera adults. Journal of applied microbiology 123, 1160–1167.
Chantawannakul, P., Puchanichanthranon, T., Wongsiri, S., 2003. Inhibitory effects of some medicinal plant extracts on the growth of Ascosphaera apis, in: III WOCMAP Congress on Medicinal and Aromatic Plants-Volume 4: Targeted Screening of Medicinal and Aromatic Plants, Economics 678. pp. 183–189.
Chapuy, C., Ribbens, L., Renou, M., Dacher, M., Armengaud, C., 2019. Thymol affects congruency between olfactory and gustatory stimuli in bees. Scientific reports 9, 1–10.
Charpentier, G., Vidau, C., Ferdy, J., Tabart, J., Vetillard, A., 2014. Lethal and sub-lethal effects of thymol on honeybee (Apis mellifera) larvae reared in vitro. Pest management science 70, 140–147.

352 Cianciosi, D., Forbes-Hernández, T.Y., Afrin, S., Gasparrini, M., Reboredo-Rodriguez, P., Manna, P.P.,
353 Zhang, J., Bravo Lamas, L., Martínez Flórez, S., Agudo Toyos, P., 2018. Phenolic compounds in
354 honey and their associated health benefits: A review. Molecules 23, 2322.
355 Colin, T., Meikle, W.G., Paten, A.M., Barron, A.B., 2019. Long-term dynamics of honey bee colonies
356 following exposure to chemical stress. Science of the Total Environment 677, 660–670.
357 Conti, B., Bocchino, R., Cosci, F., Ascrizzi, R., Flamini, G., Bedini, S., 2020. Essential oils against Varroa
358 destructor: a soft way to fight the parasitic mite of Apis mellifera. Journal of Apicultural
359 Research 59, 774–782.
360 Costa, C., Lodesani, M., Maistrello, L., 2010. Effect of thymol and resveratrol administered with candy
361 or syrup on the development of Nosema ceranae and on the longevity of honeybees (Apis
362 mellifera L.) in laboratory conditions. Apidologie 41, 141–150.
363 Cullen, M.G., Thompson, L.J., Carolan, J.C., Stout, J.C., Stanley, D.A., 2019. Fungicides, herbicides and
364 bees: A systematic review of existing research and methods. PloS one 14, e0225743.
365 Damiani, N., Fernández, N.J., Porrini, M.P., Gende, L.B., Álvarez, E., Buffa, F., Brasesco, C., Maggi,
366 M.D., Marcangeli, J.A., Eguaras, M.J., 2014. Laurel leaf extracts for honeybee pest and
367 disease management: antimicrobial, microsporicidal, and acaricidal activity. Parasitology
368 research 113, 701–709.
369 Damiani, N., Gende, L.B., Bailac, P., Marcangeli, J.A., Eguaras, M.J., 2009. Acaricidal and insecticidal
370 activity of essential oils on Varroa destructor (Acari: Varroidae) and Apis mellifera
371 (Hymenoptera: Apidae). Parasitology research 106, 145–152.
372 Dolezal, A.G., Clair, A.L.S., Zhang, G., Toth, A.L., O'Neal, M.E., 2019. Native habitat mitigates feast–
373 famine conditions faced by honey bees in an agricultural landscape. Proceedings of the
374 National Academy of Sciences 116, 25147–25155.
375 Du Plooy, W., Regnier, T., Combrinck, S., 2009. Essential oil amended coatings as alternatives to
376 synthetic fungicides in citrus postharvest management. Postharvest Biology and Technology
377 53, 117–122.
378 Ebert, T.A., Kevan, P.G., Bishop, B.L., Kevan, S.D., Downer, R.A., 2007. Oral toxicity of essential oils
379 and organic acids fed to honey bees (Apis mellifera). Journal of apicultural research 46, 220–
380 224.
381 Evans, J.D., 2003. Diverse origins of tetracycline resistance in the honey bee bacterial pathogen
382 Paenibacillus larvae. Journal of invertebrate pathology 83, 46–50.
383 Evison, S.E., 2015. Chalkbrood: epidemiological perspectives from the host–parasite relationship.
384 Current opinion in insect science 10, 65–70.
385 Ferrentino, G., Morozova, K., Horn, C., Scampicchio, M., 2020. Extraction of essential oils from
386 medicinal plants and their utilization as food antioxidants. Current pharmaceutical design 26,
387 519–541.
388 Flesar, J., Havlik, J., Kloucek, P., Rada, V., Titera, D., Bednar, M., Stropnicky, M., Kokoska, L., 2010. In
389 vitro growth-inhibitory effect of plant-derived extracts and compounds against Paenibacillus
390 larvae and their acute oral toxicity to adult honey bees. Veterinary microbiology 145, 129–
391 133.
392 Frazier, M., Mullin, C., Frazier, J., Ashcraft, S., 2008. What have pesticides got to do with it? American
393 Bee Journal 148, 521–524.
394 Fuselli, S.R., Maggi, M., de la Rosa, S.B.G., Principal, J., Eguaras, M.J., Fritz, R., 2009. In vitro
395 antibacterial and antiparasitic effect of citrus fruit essential oils on the honey bee pathogen
396 Paenibacillus larvae and the parasitic mite Varroa destructor. Journal of apicultural research
397 48, 77–78.
398 Gabriel, K.T., Kartforosh, L., Crow, S.A., Cornelison, C.T., 2018. Antimicrobial activity of essential oils
399 against the fungal pathogens Ascosphaera apis and Pseudogymnoascus destructans.
400 Mycopathologia 183, 921–934.
401 Gallai, N., Salles, J.-M., Settele, J., Vaissière, B.E., 2009. Economic valuation of the vulnerability of
402 world agriculture confronted with pollinator decline. Ecological economics 68, 810–821.

Gashout, H.A., Guzman-Novoa, E., Goodwin, P.H., Correa-Benítez, A., 2020. Impact of sublethal exposure to synthetic and natural acaricides on honey bee (Apis mellifera) memory and expression of genes related to memory. Journal of insect physiology 121, 104014.
Genersch, E., 2010. Honey bee pathology: current threats to honey bees and beekeeping. Applied microbiology and biotechnology 87, 87–97.
Goulson, D., Nicholls, E., Botías, C., Rotheray, E.L., 2015. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science 347.
Gregorc, A., Alburaki, M., Sampson, B., Knight, P.R., Adamczyk, J., 2018. Toxicity of selected acaricides to honey bees (Apis mellifera) and Varroa (Varroa destructor Anderson and Trueman) and their use in controlling varroa within honey bee colonies. Insects 9, 55.
Hallmann, C.A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Müller, A., Sumser, H., Hörren, T., 2017. More than 75 percent decline over 27 years in total flying insect biomass in protected areas. PloS one 12, e0185809.
Hýbl, M., Mráz, P., Šipoš, J., Hoštičková, I., Bohatá, A., Čurn, V., Kopec, T., 2021. Polyphenols as Food Supplement Improved Food Consumption and Longevity of Honey Bees (Apis mellifera) Intoxicated by Pesticide Thiacloprid. Insects 12, 572.
Imdorf, A., Bogdanov, S., Ochoa, R.I., Calderone, N.W., 1999. Use of essential oils for the control of Varroa jacobsoni Oud. in honey bee colonies. Apidologie 30, 209–228.
Iwasa, T., Motoyama, N., Ambrose, J.T., Roe, R.M., 2004. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, Apis mellifera. Crop protection 23, 371–378.
Jafri, H., Banerjee, G., Khan, M.S.A., Ahmad, I., Abulreesh, H.H., Althubiani, A.S., 2020. Synergistic interaction of eugenol and antimicrobial drugs in eradication of single and mixed biofilms of Candida albicans and Streptococcus mutans. AMB Express 10, 1–9.
Kloucek, P., Smid, J., Flesar, J., Havlik, J., Titera, D., Rada, V., Drabek, O., Kokoska, L., 2012. In vitro inhibitory activity of essential oil vapors against Ascosphaera apis. Natural product communications 7, 1934578X1200700237.
Korona-Glowniak, I., Glowniak-Lipa, A., Ludwiczuk, A., Baj, T., Malm, A., 2020. The in vitro activity of essential oils against Helicobacter pylori growth and urease activity. Molecules 25, 586.
Kulhanek, K., Steinhauer, N., Rennich, K., Caron, D.M., Sagili, R.R., Pettis, J.S., Ellis, J.D., Wilson, M.E., Wilkes, J.T., Tarpy, D.R., 2017. A national survey of managed honey bee 2015–2016 annual colony losses in the USA. Journal of Apicultural Research 56, 328–340.
Lal, M., Baruah, J., Begum, T., Pandey, S.K., 2020. Identification of a novel myrcene and methyl iso-eugenol rich essential oil variant (Jor Lab L-11) of lemongrass (Cymbopogon flexuosus L.). Journal of Essential Oil Bearing Plants 23, 660–668.
Li, L., Lin, Z.-G., Wang, S., Su, X.-L., Gong, H.-R., Li, H.-L., Hu, F.-L., Zheng, H.-Q., 2017. The effects of clove oil on the enzyme activity of Varroa destructor Anderson and Trueman (Arachnida: Acari: Varroidae). Saudi journal of biological sciences 24, 996–1000.
Liao, L.-H., Wu, W.-Y., Dad, A., Berenbaum, M.R., 2019. Fungicide suppression of flight performance in the honeybee (Apis mellifera) and its amelioration by quercetin. Proceedings of the Royal Society B 286, 20192041.
Llorens-Picher, M., Higes, M., Martín-Hernández, R., De la Rúa, P., Muñoz, I., Aidoo, K., Bempong, E.O., Polkuraf, F., Meana, A., 2017. Honey bee pathogens in Ghana and the presence of contaminated beeswax. Apidologie 48, 732–742.
Maggi, M.D., Ruffnengo, S.R., Gende, L.B., Sarlo, E.G., Eguaras, M.J., Bailac, P.N., Ponzi, M.I., 2010. Laboratory evaluations of Syzygium aromaticum (L.) Merr. et Perry essential oil against Varroa destructor. Journal of Essential Oil Research 22, 119–122.
Maistrello, L., Lodesani, M., Costa, C., Leonardi, F., Marani, G., Caldon, M., Mutinelli, F., Granato, A., 2008. Screening of natural compounds for the control of nosema disease in honeybees (Apis mellifera). Apidologie 39, 436–445.
Masotti, V., Juteau, F., Bessière, J.M., Viano, J., 2003. Seasonal and phenological variations of the essential oil from the narrow endemic species Artemisia molinieri and its biological activities. Journal of agricultural and food chemistry 51, 7115–7121.

Milano, F., Donnarumma, L., 2017. Determination of essential oils residues on zucchini fruits by GC-MS. Natural product research 31, 976–979.
Morales, M.M., Ramos, M.J.G., Vázquez, P.P., Galiano, F.J.D., Valverde, M.G., López, V.G., Flores, J.M., Fernández-Alba, A.R., 2020. Distribution of chemical residues in the beehive compartments and their transfer to the honeybee brood. Science of The Total Environment 710, 136288.
Mráz, P., Hýbl, M., Kopecký, M., Bohatá, A., Konopická, J., Hoštičková, I., Konvalina, P., Šipoš, J., Rost, M., Čurn, V., 2021. The Effect of Artificial Media and Temperature on the Growth and Development of the Honey Bee Brood Pathogen Ascosphaera apis. Biology 10, 431.
Nabila, B., Piras, A., Fouzia, B., Falconieri, D., Kheira, G., Fedoul, F.-F., Majda, S.-R., 2020. Chemical composition and antibacterial activity of the essential oil of Laurus nobilis leaves. Natural Product Research 1–5.
Palmer-Young, E.C., Sadd, B.M., Irwin, R.E., Adler, L.S., 2017. Synergistic effects of floral phytochemicals against a bumble bee parasite. Ecology and Evolution 7, 1836–1849.
Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., Kunin, W.E., 2010. Global pollinator declines: trends, impacts and drivers. Trends in ecology & evolution 25, 345–353.
Ramadass, M., Thiagarajan, P., 2015. Importance and Applications of Cedar oil. Research Journal of Pharmacy and Technology 8, 1714–1718.
Rinkevich, F.D., 2020. Detection of amitraz resistance and reduced treatment efficacy in the Varroa Mite, Varroa destructor, within commercial beekeeping operations. PloS one 15, e0227264.
Rios, J.-L., Recio, M.C., 2005. Medicinal plants and antimicrobial activity. Journal of ethnopharmacology 100, 80–84.
Ruoff, K., Bogdanov, S., 2004. Authenticity of honey and other bee products. Apiacta 38, 317–327.
Sahlan, M., Karwita, S., Gozan, M., Hermansyah, H., Yohda, M., Yoo, Y.J., Pratami, D.K., 2019. Identification and classification of honey's authenticity by attenuated total reflectance Fourier-transform infrared spectroscopy and chemometric method. Veterinary world 12, 1304.
Schmuck, R., Stadler, T., Schmidt, H.-W., 2003. Field relevance of a synergistic effect observed in the laboratory between an EBI fungicide and a chloronicotinyl insecticide in the honeybee (Apis mellifera L, Hymenoptera). Pest Management Science: formerly Pesticide Science 59, 279–286.
Sharma, K., Guleria, S., Razdan, V.K., Babu, V., 2020. Synergistic antioxidant and antimicrobial activities of essential oils of some selected medicinal plants in combination and with synthetic compounds. Industrial Crops and Products 154, 112569.
Smart, M.D., Otto, C.R., Lundgren, J.G., 2019. Nutritional status of honey bee (Apis mellifera L.) workers across an agricultural land-use gradient. Scientific reports 9, 1–10.
Spiltoir, C.F., 1955. Life cycle of Ascosphaera apis (Pericystis apis). American Journal of Botany 501–508.
Stevenson, P.C., 2020. For antagonists and mutualists: the paradox of insect toxic secondary metabolites in nectar and pollen. Phytochemistry Reviews 19, 603–614.
Svečnjak, L., Jović, O., Prđun, S., Rogina, J., Marijanović, Z., Car, J., Matošević, M., Jerković, I., 2019. Influence of beeswax adulteration with paraffin on the composition and quality of honey determined by physico-chemical analyses, 1H NMR, FTIR-ATR and HS-SPME/GC–MS. Food chemistry 291, 187–198.
Thompson, H.M., Fryday, S.L., Harkin, S., Milner, S., 2014. Potential impacts of synergism in honeybees (Apis mellifera) of exposure to neonicotinoids and sprayed fungicides in crops. Apidologie 45, 545–553.
Tison, L., Rößner, A., Gerschewski, S., Menzel, R., 2019. The neonicotinoid clothianidin impairs memory processing in honey bees. Ecotoxicology and environmental safety 180, 139–145.
Tock, M.L.A., Kamatou, G., Combrinck, S., Sandasi, M., Viljoen, A., 2020. A chemometric assessment of essential oil variation of three Salvia species indigenous to South Africa. Phytochemistry 172, 112249.

van den Heever, J.P., Thompson, T.S., Curtis, J.M., Ibrahim, A., Pernal, S.F., 2014. Fumagillin: an overview of recent scientific advances and their significance for apiculture. Journal of agricultural and food chemistry 62, 2728–2737.

Vázquez, P.P., Lozano, A., Uclés, S., Ramos, M.G., Fernández-Alba, A.R., 2015. A sensitive and efficient method for routine pesticide multiresidue analysis in bee pollen samples using gas and liquid chromatography coupled to tandem mass spectrometry. Journal of Chromatography A 1426, 161–173.

Vojvodic, S., Jensen, A.B., James, R.R., Boomsma, J.J., Eilenberg, J., 2011. Temperature dependent virulence of obligate and facultative fungal pathogens of honeybee brood. Veterinary Microbiology 149, 200–205.

Wiese, N., Fischer, J., Heidler, J., Lewkowski, O., Degenhardt, J., Erler, S., 2018. The terpenes of leaves, pollen, and nectar of thyme (Thymus vulgaris) inhibit growth of bee disease-associated microbes. Scientific reports 8, 1–12.

Zabka, M., Pavela, R., 2013. Antifungal efficacy of some natural phenolic compounds against significant pathogenic and toxinogenic filamentous fungi. Chemosphere 93, 1051–1056.

Zabka, M., Pavela, R., Prokinova, E., 2014. Antifungal activity and chemical composition of twenty essential oils against significant indoor and outdoor toxigenic and aeroallergenic fungi. Chemosphere 112, 443–448.

Zabka, M., Pavela, R., Slezakova, L., 2009. Antifungal effect of Pimenta dioica essential oil against dangerous pathogenic and toxinogenic fungi. Industrial Crops and Products 30, 250–253.

Zhu, L., Qi, S., Xue, X., Niu, X., Wu, L., 2020. Nitenpyram disturbs gut microbiota and influences metabolic homeostasis and immunity in honey bee (Apis mellifera L.). Environmental Pollution 258, 113671.

Zrira, S., Ghanmi, M., 2016. Chemical composition and antibacterial activity of the essential of Cedrus atlantica (Cedarwood oil). Journal of Essential Oil Bearing Plants 19, 1267–1272.

insects

MDPI

Article

Evaluating the Efficacy of 30 Different Essential Oils against *Varroa destructor* and Honey Bee Workers (*Apis Mellifera*)

Marian Hýbl [1], Andrea Bohatá [1], Iva Rádsetoulalová [2], Marek Kopecký [1], Irena Hoštičková [1], Alena Vaníčková [3] and Petr Mráz [1,*]

[1] Faculty of Agriculture, University of South Bohemia in Ceske Budejovice, Studentska 1668, 370 05 Ceske Budejovice, Czech Republic; mario.eko@seznam.cz (M.H.); bohata@zf.jcu.cz (A.B.); mkopecky@zf.jcu.cz (M.K.); jelini00@zf.jcu.cz (I.H.)
[2] Faculty of AgriSciences, Mendel University in Brno, Zemedelska 1, 613 00 Brno, Czech Republic; radsetoulalova.iva@gmail.com
[3] Aromaterapeutická KH a.s., Kšice 11, 349 01 Stříbro, Czech Republic; vanickova@karelhadek.eu
* Correspondence: mrazpe01@zf.jcu.cz

Simple Summary: Worldwide, mass losses of honey bee colonies are being observed more frequently due to Varroa mite infestation. Therefore, varroosis is considered a major problem in beekeeping participating to a large extent in colony collapse disorder. Except for direct damage of bees and suppressing their immune system caused by parasitism, Varroa mites transfer viral particles straight to bee hemolymph which can have a fatal impact. To control the mite population, several acaricidal treatments are used. Commonly used treatments are synthetic acaricides with a high risk of developing Varroa resistance population and contamination of bee products by acaricidal residues. Other commonly used treatments are organic acids, which are increasingly associated with damage of brood, adult bees, and premature deaths of queens. Therefore, in this study, we evaluated the varroacidal effect of 30 individual essential oils. The toxicity of the most effective oils selected by screening was subsequently tested on Varroa mites and adult honey bee workers simultaneously. In addition, the main components of these essential oils were specified. Several essential oils were proven to be effective against the adult female of Varroa mites and at the same dose safe for adult honey bee workers under laboratory conditions, especially manuka, peppermint, oregano, litsea, and cinnamon.

Abstract: Essential oils and their components are generally known for their acaricidal effects and are used as an alternative to control the population of the *Varroa destructor* instead of synthetic acaricides. However, for many essential oils, the exact acaricidal effect against Varroa mites, as well as the effect against honey bees, is not known. In this study, 30 different essential oils were screened by using a glass-vial residual bioassay. Essential oils showing varroacidal efficacy > 70% were tested by the complete exposure assay. A total of five bees and five mites were placed in the Petri dishes in five replications for each concentration of essential oil. Mite and bee mortality rates were assessed after 4, 24, 48, and 72 h. The LC_{50} values and selectivity ratio (SR) were calculated. For essential oils with the best selectivity ratio, their main components were detected and quantified by GC-MS/MS. The results suggest that the most suitable oils are peppermint and manuka (SR > 9), followed by oregano, litsea (SR > 5), carrot, and cinnamon (SR > 4). Additionally, these oils showed a trend of the increased value of selective ratio over time. All these oils seem to be better than thymol (SR < 3.2), which is commonly used in beekeeping practice. However, the possible use of these essential oils has yet to be verified in beekeeping practice.

Keywords: acaricidal effect; complete exposure bioassay; honey bee; screening; Varroa mite

Citation: Hýbl, M.; Bohatá, A.; Rádsetoulalová, I.; Kopecký, M.; Hoštičková, I.; Vaníčková, A.; Mráz, P. Evaluating the Efficacy of 30 Different Essential Oils against *Varroa destructor* and Honey Bee Workers (*Apis mellifera*). *Insects* **2021**, *12*, 1045. https://doi.org/10.3390/insects12111045

Academic Editors: Ivana Tlak Gajger and Franco Mutinelli

Received: 2 November 2021
Accepted: 19 November 2021
Published: 21 November 2021

1. Introduction

The main threat for beekeeping is a varroosis caused by the obscure ectoparasitic mite *Varroa destructor* Anderson and Trueman (Acari: Varroidae) [1,2]. The mite feeds on the fat body of bees [3] and thus reduces the weight and fitness of newly emerging adult bees, affects cuticle properties [4] and suppresses the immune response system [5]. In addition, *V. destructor* acts as a vector of viruses [6], including deformed wing virus, Kashmir bee virus, and Israeli acute paralysis virus [7–11]. These viruses are transmitted in large doses directly to the hemolymph of the bee brood and adult honey bees [5]. Infected individuals weaken, their lifespan is shorter, and the infection can lead through visible damaged bodies and wings [12,13] to the colony collapse at the final stage [1,6]. For these reasons, and also due to its almost worldwide distribution [1], *V. destructor* is associated with colony collapse disorder (CCD) [14,15].

Reproduction of the *V. destructor* mite is closely related and synchronized with the development of the bee brood [16]. Adult mated female mites enter the bee colony attached to worker and drone bees, usually hidden under the sternites of bees, and then enter brood cells only several hours before capping. Varroa mites can find the adult honey bee workers and bee brood before capping based on chemical communication [1]. In colonies highly infested (>7%) with *V. destructor* [1], the bee population is significantly reduced, and eventually, the entire colony crashes unless the mite population is treated [17]. Colonies in temperate areas must therefore be treated several times in a year against *V. destructor* to keep mite populations at acceptable levels [18].

For a long time, the use of synthetic chemicals has been considered the most effective way to control *V. destructor* [19], especially pyrethroids and organophosphates [20]. Except for their declining efficiency due to emerging resistance against *V. destructor* [21–23], excessive use of these compounds has, in many cases, also led to contamination of bee products [24–26], especially honey and beeswax [24]. This could endanger the health of bees and humans with potential sublethal doses of pesticide residue mixtures [27,28]. As a result, the idea of finding new and safer ways to control the parasite is spreading. Thus, natural products offer a very desirable alternative to synthetic products. Interest in these substances is still growing because they are generally cheap and have lower health risks for humans and bees [29].

In response, beekeepers are showing a growing interest in treatments that work on physical intolerance rather than enzyme degradation, as is the case with synthetic acaricides, to which resistance develops. Therefore, natural chemicals such as organic acids, essential oils, and their derivatives are increasingly used [30,31]. However, several studies suggest that the use of organic acids against Varroa may be harmful to bees. For example, damage and removal of open and capped brood are most commonly observed [32,33]. In addition, permanent damage to the digestive and excretory organs and glands of bees was described [34,35], as well as damage to the queen or often even premature death [36,37], or a decrease in the pH of honey during the following season [38].

Another possible way to reduce Varroa mites is essential oils (EO) [39]. According to The Commission of the European Pharmacopoeia, EOs are odorous products, usually with a complex composition, obtained from a botanically defined plant raw material by steam distillation, dry distillation, or a suitable mechanical process without heating. They are usually separated from the aqueous phase by a physical process that does not significantly affect their composition. EOs are lipophilic and may contain over 100 different plant secondary metabolites (terpenoids and phenylpropanoids, monoterpenes, sesquiterpenes, aldehydes, alcohols, etc.) [40]. Among natural substances, essential oils represent one of the most promising alternatives to synthetic chemicals [41–48], with minimal side effects [49–52]. The effectiveness of EOs against *V. destructor* is comparable to organic acids, but the application of EOs causes a lesser degree of stress in bees than the application of organic acids [29].

In addition to acaricidal effects, the application of EOs into hives often also causes antimicrobial effects, which can lead to an overall improvement in the health status of honey

bee colonies [53]. Most research suggests that essential oils may be a useful alternative to maintaining a low level of mite infestation in hives [39,54–57]. Adamczyk et al. [58] concluded that the presence of residues of essential oil components in honey samples does not pose a hygiene risk or a risk to human health.

Despite the promising acaricidal effects of various EOs found in vitro [54,55,57], only a fraction of them has been tested under beehive conditions [39]. This could be the reason why EOs have not yet been included in many commercial formulations, with the exception of some cases [53].

The aim of the study was therefore to determine the acaricidal effect of a large number of selected EOs against Varroa mites, as well as their effect on honey bees in vitro, which select the most promising essential oils for the in vivo experiments. In addition, the most promising EOs were quantified for their major components.

2. Materials and Methods

2.1. Biological Material and Essential Oils

V. destructor mites and honey bees (*Apis mellifera*) used in this study were obtained from the experimental apiary of the Faculty of Agriculture, the University of South Bohemia in České Budějovice, (Czech Republic). To rear mites, 4 honey bee colonies were infested by Varroa mites and untreated for over 12 months. From the infested beehives, the bees were collected in a mesh container by sweeping from the brood frames and subsequently exposed to CO_2. After anesthesia of the bees, the vessel was closed and shaken until mites fell over the mesh bottom [59]. Thus, a sufficient number of adult vital female mites were collected. Mites showing signs of defect, newly molded, or poorly mobile were eliminated.

A total of 30 essential oils (EO) were obtained from company 1. Aromaterapeutická KH a.s. (Czech Republic). The list of EOs, their abbreviations, Latin names, and part of used plants are given in Table 1.

Table 1. The list of essential oils, abbreviations, Latin names, and part of the used plants.

English Name	Abbreviation	Latin Name	Part of Plant
Black pepper	PEP	*Piper nigrum*	berry
Blue chamomile	BCH	*Matricaria chamomilla*	flower
Carrot	CAT	*Daucus carota*	seeds
Cinnamon	CIN	*Cinnamomum zeylanicum*	bark
Clove Bud	CB	*Eugenia caryophyllata*	leaves, buds, and twigs
Copaiba	COP	*Copaifera reticulata*	resin
Coriander	COR	*Coriandrum sativum*	seeds
Fennel	FEN	*Foeniculum vulgare*	seeds
Ginger	GIN	*Zingiber officinale*	rhizome
Green cardamom	CAR	*Elettaria cardamomum*	seeds
Laurel	LAU	*Laurus nobilis*	leaves
Lavender	LAV	*Lavandula angustifolia*	flowering herb
Litsea	LIT	*Litsea cubeba*	fruits
Mace	MAC	*Myristica fragrans*	flower
Manuka	MAN	*Leptospermum scoparium*	leaves and twigs
Maroc chamomile	MCH	*Ormenis multicaulis*	herb
Nutmeg	NUT	*Myristica fragrans*	seeds
Oregano	ORG	*Origanum vulgare*	herb
Pelargonium	PEL	*Pelargonium graveolens*	leaves and flowers
Peppermint	PPM	*Mentha piperita*	herb
Ravensara	RAV	*Ravensara aromatica*	leaves and twigs
Roman chamomile	RCH	*Anthemis nobilis*	flower
Rosemary	ROS	*Rosmarinus officinalis*	herb
Sage	SAG	*Salvia officinalis*	leaves
Savory	SAV	*Satureja montana*	herb
Spearmint	SPM	*Mentha spicata crispa*	flowering herb

Table 1. *Cont.*

English Name	Abbreviation	Latin Name	Part of Plant
Thyme	TYM	*Thymus vulgaris*	herb
Turmeric	TUR	*Curcuma longa*	root
Wild thyme	WTYM	*Thymus serpyllum*	herb
Wormwood	WW	*Artemisa absinthium*	herb

2.2. Screening of Essential Oils for Their Acaricidal Activity

To evaluate EO acute toxicity on *V. destructor*, a glass-vial residual bioassay was used [60]. Each tested product was diluted in acetone (0.375 µL EO/500 µL acetone). This solution was pipetted into a 10 mL glass vial. Glass vials were rolled on their side until the acetone evaporated and EOs created a cohesive film. Then, 5 vital female adult mites were placed in each glass vial using a fine brush. The glass vials were sealed and placed in a dark room at 25 °C and 65% RH. For each treatment, including acetone as a negative control and thymol (THM) as a positive control; 5 repetitions were provided (each repetition in an individual glass vial).

The mortality rates of Varroa mites were evaluated 2 and 4 h after the treatment, and the efficacy of tested EOs was determined [55]. The mites were transferred to a white pad and encouraged to move with the brush. Mites that did not move even after repeated brushing were considered dead.

2.3. Complete Exposure Bioassay

EOs showing >70% mite mortality in the screening test were subjected to further testing in the complete exposure method [61]. Dosages of EOs were prepared based on the mortality of previous experiments with honey bees (data not included). A selected amount of EOs was diluted in 0.5 mL of acetone. This solution was pipetted on the bottom of the Petri dish and subsequently covered with filter paper (Whatman 1). After evaporation of the solvent, five vital adult honey bee workers were placed in each Petri dish, together with five vital female adult Varroa mites. Positive control (thymol) and negative control (acetone only) were included. Altogether, 5 replicates were established for each treatment (each repetition in an individual Petri dish). Immediately after the establishment, the Petri dishes were transferred to an incubator (28 °C ± 0.5). Honey bee and mite mortality were assessed after 4, 24, 48, and 72 h. The values of LC_{50} and selectivity ratio (SR) were calculated. SR is a ratio between mite and bee toxicity, and it was determined according to the following formula: $SR = LC_{50\ A.\ mellifera}/LC_{50\ V.\ destructor}$.

2.4. Assessment of the Main Components of the Examined EOs

Samples of essential oils were analyzed diluted 1:10,000 in hexane by GC MS/MS system consisting of TriPlus autosampler, Trace GC Ultra gas chromatograph equipped with a TG-5MS fused silica capillary column, 30 m × 0.25 mm × 0.25 µm and coupled to a mass spectrometer TSQ Quantum XLS all from Thermo Fischer Scientific, Cleveland, OH, USA. Helium was used as a carrier gas at 1.0 mL/min. A total of 1 µL of the sample was injected into the SSL injector in the splitless mode set at 280 °C. The oven temperature was programmed as follows: start at 40 °C and held for 5 min, then increased to 150 °C at a rate of 3 °C/min and held for 0.5 min, then increased to 250 °C at a rate of 10 °C/min, then increased to 290 °C at a rate of 25 °C, and finally maintained at 290 °C for 10 min. The temperature of the transfer line was held at 250 °C, and the ion source was operating at 200 °C. TIC mode was performed on Q1 at 70 eV of ionization energy and mass range 50–450 m/z. To exclude congestion of detector the scanning was performed after 6 min of injection. The data were processed in Thermo Xcalibur 3.0.63 (Thermo Fisher, Waltham, MA, USA). Component identification was made based on comparison with the NIST Mass Spectral Search Program library v 2.0 f (Thermo Fisher). The quantification was achieved based on Q3 SIM mode focused on fragmentation ions of desired compounds and also via

an external calibration curve. The Thujone (Sigma Aldrich, St. Louis, MO, USA) was used as an internal and also external standard.

2.5. Statistical Analyses

Statistical analyses of the screening of essential oils, including graphical outputs, were processed in STATISTICA (version 14, TIBCO Software Inc., Palo Alto, CA, USA, 2021), specifically, the analysis of variance procedure ANOVA, preceded by a normality test. Statistical significance was tested at a level of significance = 0.05.

Probit analyses were calculated in XLSTAT (Addinsoft, 2016) incorporating natural mortality into the analyses. The concentration of essential oils was transformed logarithmically. LD_{50} with 95% confidence intervals ($p < 0.05$) were fitted.

The in vitro effect of each active substance on mortality of both Varroa mite and honey bees was analyzed by the test of hypothesis for two samples representing independent binomial experiments, and the acaricidal effects of active substances were subsequently evaluated (GenStat 17). Significant differences among substances were stated where $p \leq 0.05$.

3. Results

3.1. Screening of Essential Oils for Their Acaricidal Activity

All 30 EOs were screened for acaricidal effect in glass vials (Figure 1). Based on these results, the EOs were divided into three categories according to their efficacy. A total of 11 EOs showed a high acaricidal efficacy (>70%) and were further tested on Petri dishes (complete exposure assay) simultaneously with honey bees and mites. These were MAN, TYM, WTYM, ORG, SAV, CIN, CB, PPM, CAT, PEL, LIT, and THM as a positive control. The category of moderately effective oils (30–50%) includes ROS, RAV, TUR, RCH, LAV, CAR, PEP, and GIN. The last category with an efficiency of less than 30% includes NUT, FEN, MAC, BCH, MCH, COP, SAG, SPM, COR, LAU, and WW. Oils showing less than 70% efficacy were further tested.

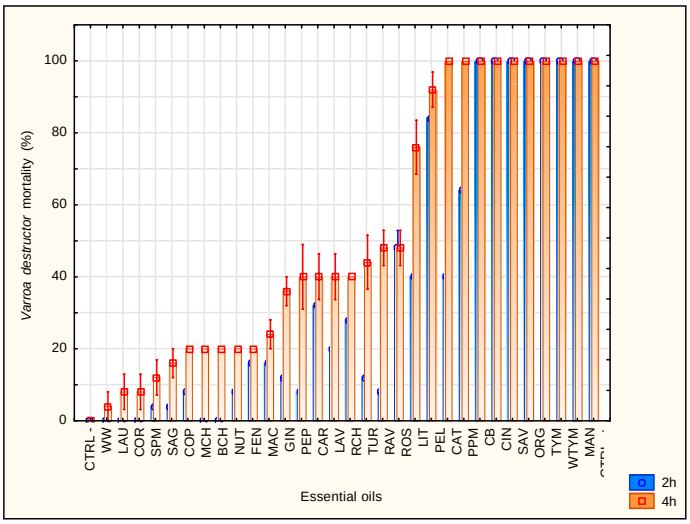


Figure 1. Mortality rates of *Varroa destructor* in glass vial bioassay after 2 and 4 h of EO exposition. The error bars denote standard deviation. Full name of each abbreviation is shown in Table 1.

3.2. Complete Exposure Bioassay

The complete exposure bioassay reveals that EO from MAN showed by far the lowest LC_{50} value against Varroa mites both after 4 and 72 h of exposure. EOs from TYM, ORG, and control THM also had a low LC_{50} value after 72 h of exposure. A moderate LC_{50} value after 72 h showed PPM, SAV, WTYM, CB, and CIN. EO from PEL and CAT showed a relatively high value. The lowest LC_{50} value for bees after 72 h of exposure had EOs from

MAN, TYM, and control THM, slightly high values had CB and ORG. In contrast, bees were most tolerant of EOs from CAT, PPM, LIT, and PEL (Table 2).

Table 2. Complete exposure bioassay. LC_{50} (µL) of essential oils on *V. destructor* and *A. mellifera* and their selectivity ratio in a monitored period. Green highlighting means low value of selectivity ratio (<3), yellow highlighting means moderate value of selectivity ratio (3–5), and red highlighting means high value of selectivity ratio (>5).

EO	Species	4 h			24 h			48 h			72 h		
		LC_{50}	95% CL		LC_{50}	95% CL		LC_{50}	95% CL		LC_{50}	95% CL	
THM	*V. destructor*	1.505	1.180	1.937	0.834	0.629	1.052	0.660	0.475	0.846	0.660	0.475	0.846
	A. mellifera	6.181	5.074	7.847	4.090	3.189	6.759	2.427	2.097	2.871	2.112	1.940	2.320
	Selectivity ratio	4.107			4.903			3.675			3.198		
CAT	*V. destructor*	10.449	6.806	34.882	4.167	2.457	6.630	3.276	1.930	4.590	2.539	1.187	3.653
	A. mellifera	18.607	13.845	64.136	13.048	9.588	27.137	11.557	8.855	19.447	11.557	8.855	19.447
	Selectivity ratio	1.781			3.131			3.527			4.552		
PPM	*V. destructor*	8.121	6.159	13.576	2.512	1.430	3.578	1.732	0.499	2.806	1.066	0.011	2.197
	A. mellifera	12.951	11.259	14.994	10.759	9.483	12.156	10.285	9.109	11.568	10.285	9.109	11.568
	Selectivity ratio	1.595			4.283			5.939			9.651		
SAV	*V. destructor*	3.825	3.165	4.918	2.008	1.323	2.754	1.459	0.626	2.075	1.364	0.417	1.996
	A. mellifera	11.657	9.247	16.335	5.786	4.607	7.218	5.275	4.273	6.467	4.621	3.884	5.897
	Selectivity ratio	3.048			2.881			3.615			3.386		
WTYM	*V. destructor*	8.185	5.218	22.390	2.549	1.495	8.207	2.013	0.926	7.327	1.861	0.825	5.487
	A. mellifera	9.074	8.106	10.780	7.517	6.606	8.265	6.512	5.958	7.494	6.250	5.603	6.897
	Selectivity ratio	1.109			2.949			3.236			3.358		
ORG	*V. destructor*	3.517	2.339	7.322	0.879	0.638	1.302	0.577	0.280	0.924	0.577	0.280	0.924
	A. mellifera	6.982	6.136	7.889	3.362	2.997	3.803	3.362	2.997	3.803	3.362	2.997	3.803
	Selectivity ratio	1.985			3.827			5.830			5.830		
PEL	*V. destructor*	2.798	0.113	4.758	2.291	0.247	3.825	2.402	0.804	3.532	2.272	0.788	3.311
	A. mellifera	17.122	13.427	27.935	12.401	10.159	17.209	9.479	8.132	11.201	9.479	8.132	11.201
	Selectivity ratio	6.120			5.413			3.945			4.171		
MAN	*V. destructor*	1.262	0.848	3.192	1.029	0.558	2.880	0.265	0.020	0.540	0.158	0.011	0.497
	A. mellifera	1.975	1.662	2.681	1.415	1.218	1.666	1.472	1.277	1.720	1.472	1.277	1.720
	Selectivity ratio	1.565			1.375			5.551			9.333		
LIT	*V. destructor*	4.801	3.522	7.436	2.716	1.322	4.311	2.116	0.243	3.761	1.807	0.243	2.989
	A. mellifera	11.660	9.524	15.222	11.590	8.994	20.096	9.207	7.721	12.115	9.678	7.255	18.278
	Selectivity ratio	2.429			4.267			4.352			5.354		
TYM	*V. destructor*	1.279	0.985	1.613	0.678	0.314	0.940	0.678	0.314	0.940	0.587	0.202	0.851
	A. mellifera	8.759	6.684	14.553	3.887	3.295	4.837	3.113	2.696	3.763	2.677	2.418	2.982
	Selectivity ratio	6.848			5.731			4.590			4.557		
CB	*V. destructor*	2.337	1.829	2.962	1.690	1.237	2.143	1.490	1.207	1.776	1.490	1.207	1.776
	A. mellifera	5.965	4.620	10.868	4.860	4.023	6.546	3.305	2.790	4.179	3.305	2.790	4.179
	Selectivity ratio	2.553			2.875			2.218			2.218		
CIN	*V. destructor*	4.321	3.163	5.979	2.820	1.577	4.002	2.529	1.370	3.590	1.543	0.829	2.484
	A. mellifera	10.635	9.559	11.972	7.488	6.680	8.408	7.488	6.680	8.408	7.007	5.835	8.664
	Selectivity ratio	2.461			2.655			2.960			4.542		

The selectivity ratio was calculated based on the LC_{50} values. The estimated LC_{50} values, including standard deviation obtained at each observation time and selectivity ratio for every treatment, are shown in Table 2. By far, the highest value of the selective ratio was reached after 72 h of exposure to EO from PPM (SR = 9.65) and MAN (SR = 9.33). ORG (SR = 5.83) and LIT (SR = 5.35) also reached high values at 72 h. All these four oils had an increasing SR value over time. In contrast, TYM and PEL oils had the highest SR value after 4 h of exposure (SR = 6.85; SR = 6.12). A significant decrease in this value was observed in the following measurements. Moderately high SR values were observed after 72 h of exposure in CAT, SAV, and WTYM, which showed an increasing tendency of SR value in time (SR = 4.55; SR = 3.39; SR = 3.36). A moderate-to-high SR value was also observed in THM (positive control). After 4 h of experiment, THM showed even one of the highest SR

values (SR = 4.11), however, with a declining trend of SR values in time. A constantly low value of SR was observed with CB, as in each measurement SR was less than 3. Similarly, CIN also had a low value of SR, and with the exception of the last measurement after 72 h of exposure, the level of SR increased significantly (SR = 4.54).

The main components and their quantity of the most effective EOs were assessed (Table 3). The most frequent substances were carvacrol and p-cymene.

Table 3. Composition of the most effective essential oils and their constituents' quantity (>5%).

EO	Main Components and Their Quantity (%)				
Carrot	Ceratol 30.28	α-Pinen 15.462	Sabinen 10.22	β- Caryophyllen 8.31	β-bisabolen 5.63
Peppermint	Limonen 38.02	Menthol 16.41	α-Pinen 15.92	β-Pinen 11.46	Menthon 5.65
Savory	Carvacrol 41.67	γ-Terpinen 35.82	p-Cymen 11.73	-	-
Wild thyme	Thymol 16.33	Carvacrol 15.38	p-Cymen 15.01	Geraniol 10.62	γ-Terpinen 10.30
Oregano	Carvacrol 73.50	p-Cymen 6.97	γ-Terpinen 6.02	-	-
Pelargonium	Citronellol 33.51	Geraniol 15.36	Citronellylformiat 7.81	Isomenthon 5.61	10-epi-g-Eudesmol 5.37
Manuka	Calamenene 17.92	Leptospermone 16.02	Flaveson 5.95	α-Selinene 4.62	-
Litsea	Citral A 39.03	Citral B 29.35	Limonen 13.74	-	-
Thyme	Thymol 40.96	p-Cymen 16.76	-	-	-
Clove Bud	Eugenol 86.62	β-Caryophyllen 10.21	-	-	-
Cinnamon	trans-Cinnamaldehyde 77.69	Eugenol 7.50	-	-	-

4. Discussion

Investigation of the acaricidal activity of essential oils is a major concern of many scientific studies. However, large-scale screening of a number of EOs is rare, and most of the effort is devoted to an individual or a small number of selected oils, such as thyme, clove bud, or oregano [55,56,62]. In this study, the acaricidal effect of 30 EOs on *V. destructor* mites was assessed by the glass vials bioassay (Figure 1), which represents a simple and quick way to determine the effectiveness of individual EOs [60].

Thymol, as a derivate of thyme, was included in the screening as a positive control, as it is commonly used in beekeeping practice as an acaricide [63]. However, thymol could have some negative effects on bees, including toxicity on bee brood, metabolic disorders, changes in bee's behaviors, etc. [64–71].

In the experiment, after 4 h of exposure, all EOs showed either the same or higher acaricidal effect than after 2 h. Based on the results of mortality after 4 h of exposure, the individual EOs were divided into three categories according to their effectiveness: highly effective, moderately effective, and minimally effective. The oils in the highly effective group, including MAN, WTYM, TYM, ORG, SAV, CIN, CB, PPM, CAR, PEL, and LIT, were further tested. Almost all oils in this group were able to kill 100% of mites after 2 h, with the exception of PPM, CAR, PEL, and LIT. The EOs from the moderately effective group have still the potential to participate in the mite control; however, a higher dose or applying a certain mixture showing a stronger synergistic effect would be needed. From the group of moderately effective EOs, the best acaricidal activity belonged to ROS, RAV, and TUR. The oils from the minimally effective group showed a very low varroacidal effect, and therefore, they were not suitable for further testing. Especially WW, LAU, and COR appear to be ineffective.

The 11 EOs from the highly effective group were further tested in order to determine the most suitable EOs for the best potential use in beekeeping practice. In addition to mite toxicity, the bee tolerance was necessary to be evaluated. Therefore, the method of complete exposure assay [61] was chosen, which allows the evaluation of selectivity ratio (SR), the most telling data for this purpose, in addition to LC for mites and bees [57].

In the complete exposure bioassay, after 4 h of exposure, only MAN and TYM showed a higher level of mite toxicity than THM (control). After 72 h of exposure at the end of the experiment, MAN, TYM, and ORG showed higher mite toxicity. The higher degree of

toxicity of the above-mentioned EOs, compared with THM, is probably due to the content of other active substances (carvacrol, p-cymene, calamenene, leptospermone), which can additionally act synergistically [56,72]. While the varroacidal effect has already been described for TYM and ORG [55,56,62], for MAN, it has not been described yet. However, its antimicrobial and also acaricidal effects against other mite species (*Dermatophagoides* and *Tyrophagus*) have been observed [73,74]. Regarding bee toxicity, only EOs from CB and MAN were more toxic than THM after 4 h of exposure. After 72 h, at the end of the experiment, a higher degree of toxicity was observed only in EO from MAN. Thus, the results indicate higher toxicity of THM to Varroa mites but also to honey bees [55,57].

The ratio between mite and bee toxicity is defined as selectivity ratio (SR) values. At the beginning of the experiment, after 4 h of exposure, THM showed an SR value of 4.107, which was better than most EOs tested. Higher SR value was observed only at PEL (6.120) and TYM (6.848). However, with the duration of exposure, the SR value of THM decreased. After 72 h of exposure, the value was only 3.198. This can be explained by a decrease in mite toxicity, an increase in bee toxicity, or a combination of both in time. [64,75]. A similar trend was observed for PEL and TYM. In both EOs, the SR value also decreased with the duration of exposure; however, in both EOs, the SR value was always higher, compared with THM. This declining trend in the SR value with increasing exposure time for TYM is consistent with the results of Damiani et al. [62] and is probably due to the high thymol content that is characteristic of thyme [76]. This declining trend in the SR value indicates the potential unsuitability of EOs with these properties, and these EOs need to be subjected to further testing.

Stable to slightly fluctuating development of SR values depending on the duration of exposure was observed at SAV and CB. The initial values at the beginning of the measurement were very similar to the values at the end of the experiment and do not change significantly during the experiment. However, the SR value was significantly lower in CB than in THM, which is in accordance with the results of Damiani et al. [62], and in the case of SAV, the SR values are similar to THM. In the other tested EOs, an opposite trend was observed, and the SR increased with the time of exposure.

The best SR value after 72 h was determined at EOs from PPM (SR = 9.651) and MAN (SR = 9.333), followed by ORG (SR = 5.830) and LIT (SR = 5.354). From the results of Nazer and Al-Abbadi [77], it seems EO from PPM is more suitable to control varroosis than THM in vivo. The same conclusion can be drawn from the results of Damiani et al. [62] in the case of ORG in in vitro conditions. There is still a lack of varroacidal data from MAN and LIT in the literature; however, a strong antimicrobial effect against *Clostridium*, *Bifidobacterium*, *Escherichia*, *Staphylococcus*, *Lactobacillus*, and an acaricidal effect against *Dermatophagoides* and *Tyrophagus* is known for both EOs [73,74,78].

A very good result after 72 h was also observed at EOs from CIN (SR = 4.542) and CAT (SR = 4.552). CIN is proposed as a suitable option for reducing the population of *V. destructor*. In addition, CIN has a strong repellent effect on *V. destructor* mites and is also gentle on bees [39]. The suitability of CAT for further testing in the beehive conditions is also proved by its strong inhibitory effect against *Ascosphera apis* and *Paenibacillus larvae* [79,80]. A slightly lower SR value, but still higher than THM, was observed in EO from WTYM (SR = 3.358).

Since the chemical composition of EOs is influenced by many factors (geographical origin, part of the plant, agrotechnics, genotype, extraction technology, etc.), it is necessary to know their composition to interpret the effect of individual EOs [78].

According to SR of EOs from PPM and MAN, they seem to be the most promising oils against *V. destructor*. The most represented substances in PPM were limonene, menthol, and α-pinene. Limonene has been shown to be effective in reducing the population of *V. destructor* at a colony level [81] and has strong antimicrobial effects [82]. Varoacidal [55] and antimicrobial effects have also been reported for menthol [83], whereas α-pinene is known for its inhibitory effects on bacteria [84]. In addition, it can also be produced in larger quantities by genetically modified bacteria [85]. In the case of MAN, calamenene

and leptospermone were the most abundant constituents. Celemonene-containing oils show high antimicrobial and fungicidal activity and are effective against a wide range of pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) strains, and also have high antioxidant activity [86]. Leptospermone is known for its bactericidal, antiviral, and acaricidal effects [73,74].

Other EOs with suitable results were LIT and ORG. The main components of LIT were citral (A and B) and limonene. The findings of Liu et al. [87] agree with ours that citral is the main component of litsea and has a strong aroma and strong antimicrobial effects [88] against both, gram-positive and gram-negativebacteria [78]. At ORG, carvacrol was absolutely dominant and is known for its significant acaricidal and antimicrobial effects. In addition, it also has anti-inflammatory and antimutagenic, and antigenotoxic effects [89]. CIN and CAT also showed a significant acaricidal effect. The main component of CIN was cinnamaldehyde, to which Conti et al. [39] attributed the main varroacidal effects in cinnamon EO. It also has antibacterial effects [90]. Ceratol and α-pinen were predominant in CAT. The last EO with better results than THM was WTYM, with an almost balanced representation of thymol, carvacrol, and p-cymene.

5. Conclusions

The results based on selectivity ratio (SR) value for individual EOs showed that potential best EOs for Varroa control are PPM and MAN, followed by ORG and LIT. Other suitable candidates seem to be CAT, SAV, WTYM, and CIN. All these oils showed better SR values at the end of the experiment than THM (control group), which is used in beekeeping practice. Additionally, these oils showed a trend of an increased value of the selective ratio.

Thymol showed very good SR at the beginning of the experiment, but this value declined with all following measurements. At the end of the experiment, the SR value was lower than the values of most tested essential oils. This trend was also observed in EOs from PEL and TYM.

Except for well-known substances such as thymol, menthol, and carvacrol, other components appear to be potentially interesting for the control of Varroa, especially citral, limonene, calamenene, leptospermone, p-cymene, and cinnamaldehyde, as the main compounds of the most effective EOs.

Author Contributions: Conceptualization, M.H., M.K. and P.M.; data curation, A.B., I.R. and I.H.; investigation, M.K., I.H., A.V. and P.M.; methodology, M.H.; supervision, A.B. and A.V.; writing—original draft preparation, M.H. and P.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The study did not report any data.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Rosenkranz, P.; Aumeier, P.; Ziegelmann, B. Biology and control of *Varroa destructor*. *J. Invertebr. Pathol.* **2010**, *103*, S96–S119. [CrossRef]
2. Sammataro, D.; Gerson, U.; Needham, G. Parasitic mites of honey bees: Life history, implications, and impact. *Annu. Rev. Entomol.* **2000**, *45*, 519–548. [CrossRef] [PubMed]
3. Ramsey, S.D.; Ochoa, R.; Bauchan, G.; Gulbronson, C.; Mowery, J.D.; Cohen, A.; Lim, D.; Joklik, J.; Cicero, J.M.; Ellis, J.D. *Varroa destructor* feeds primarily on honey bee fat body tissue and not hemolymph. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 1792–1801. [CrossRef]
4. Bowen-Walker, P.L.; Gunn, A. The effect of the ectoparasitic mite, *Varroa destructor* on adult worker honeybee (*Apis mellifera*) emergence weights, water, protein, carbohydrate, and lipid levels. *Entomol. Exp. Appl.* **2001**, *101*, 207–217. [CrossRef]
5. Yang, X.; Cox-Foster, D.L. Impact of an ectoparasite on the immunity and pathology of an invertebrate: Evidence for host immunosuppression and viral amplification. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 7470–7475. [CrossRef] [PubMed]

6. Le Conte, Y.; Ellis, M.; Ritter, W. Varroa mites and honey bee health: Can varroa explain part of the colony losses? *Apidologie* **2010**, *41*, 353–363. [CrossRef]
7. Bowen-Walker, P.; Martin, S.; Gunn, A. The transmission of deformed wing virus between honeybees (*Apis mellifera* L.) by the ectoparasitic mite *Varroa jacobsoni* Oud. *J. Invertebr. Pathol.* **1999**, *73*, 101–106. [CrossRef]
8. Chen, Y.; Zhao, Y.; Hammond, J.; Hsu, H.; Evans, J.; Feldlaufer, M. Multiple virus infections in the honey bee and genome divergence of honey bee viruses. *J. Invertebr. Pathol.* **2004**, *87*, 84–93. [CrossRef]
9. Di Prisco, G.; Pennacchio, F.; Caprio, E.; Boncristiani, H.F., Jr.; Evans, J.D.; Chen, Y. *Varroa destructor* is an effective vector of Israeli acute paralysis virus in the honeybee, *Apis mellifera*. *J. Gen. Virol.* **2011**, *92*, 151–155. [CrossRef]
10. Martin, S.J.; Highfield, A.C.; Brettell, L.; Villalobos, E.M.; Budge, G.E.; Powell, M.; Nikaido, S.; Schroeder, D.C. Global honey bee viral landscape altered by a parasitic mite. *Science* **2012**, *336*, 1304–1306. [CrossRef]
11. Shen, M.; Cui, L.; Ostiguy, N.; Cox-Foster, D. Intricate transmission routes and interactions between picorna-like viruses (Kashmir Bee Virus and Sacbrood Virus) with the honeybee host and the parasitic varroa mite. *J. Gen. Virol.* **2005**, *86*, 2281–2289. [CrossRef] [PubMed]
12. Genersch, E. Honey bee pathology: Current threats to honey bees and beekeeping. *Appl. Microbiol. Biotechnol.* **2010**, *87*, 87–97. [CrossRef]
13. Meixner, M.D. A historical review of managed honey bee populations in Europe and the United States and the factors That may affect them. *J. Invertebr. Pathol.* **2010**, *103*, S80–S95.
14. Annoscia, D.; Del Piccolo, F.; Nazzi, F. How does the mite *Varroa destructor* kill the honeybee *Apis mellifera*? Alteration of cuticular hydrcarbons and water loss in infested honeybees. *J. Insect Physiol.* **2012**, *58*, 1548–1555. [CrossRef]
15. Potts, S.G.; Biesmeijer, J.C.; Kremen, C.; Neumann, P.; Schweiger, O.; Kunin, W.E. Global pollinator declines: Trends, impacts and drivers. *Trends Ecol. Evol.* **2010**, *25*, 345–353. [CrossRef] [PubMed]
16. Martin, S.J. Ontogenesis of the mite *Varroa jacobsoni* Oud. in worker brood of the honeybee *Apis mellifera* L. under natural conditions. *Exp. Appl. Acarol.* **1994**, *18*, 87–100. [CrossRef]
17. Murilhas, A.M. *Varroa destructor* infestation impact on *Apis mellifera* Carnica capped worker brood production, bee population and honey storage in a Mediterranean climate. *Apidologie* **2002**, *33*, 271–281. [CrossRef]
18. Delaplane, K.S.; Hood, W.M. Effects of delayed acaricide treatment in honey bee colonies parasitized by *Varroa jacobsoni* and a late-season treatment threshold for the South-Eastern USA. *J. Apic. Res.* **1997**, *36*, 125–132. [CrossRef]
19. Meikle, W.G.; Sammataro, D.; Neumann, P.; Pflugfelder, J. Challenges for developing pathogen-based biopesticides against *Varroa destructor* (Mesostigmata: Varroidae). *Apidologie* **2012**, *43*, 501–514. [CrossRef]
20. Calderone, N.W.; Shimanuki, H.; Allen-Wardell, G. An in vitro evaluation of botanical compounds for the control of the honeybee pathogens *Bacillus larvae* and *Ascosphaera apis*, and the secondary invader *B. Alvei*. *J. Essent. Oil Res.* **1994**, *6*, 279–287. [CrossRef]
21. Cakmak, I.; Aydin, L.; Gulegen, E.; Wells, H. Varroa (*Varroa destructor*) and tracheal mite (*Acarapis woodi*) incidence in the Republic of Turkey. *J. Apic. Res.* **2003**, *42*, 57–60. [CrossRef]
22. Price, K.L.; Lummis, S.C. An atypical residue in the pore of *Varroa destructor* GABA-activated RDL receptors affects picrotoxin block and thymol modulation. *Insect Biochem. Mol. Biol.* **2014**, *55*, 19–25. [CrossRef] [PubMed]
23. Rinkevich, F.D. Detection of amitraz resistance and reduced treatment efficacy in the varroa mite, *Varroa destructor*, within commercial beekeeping operations. *PLoS ONE* **2020**, *15*, e0227264. [CrossRef]
24. Wallner, K. Varroacides and their residues in bee products. *Apidologie* **1999**, *30*, 235–248. [CrossRef]
25. Kanga, L.H.; Jones, W.A.; Gracia, C. Efficacy of strips coated with *Metarhizium anisopliae* for control of *Varroa destructor* (Acari: Varroidae) in honey bee colonies in Texas and Florida. *Exp. Appl. Acarol.* **2006**, *40*, 249–258. [CrossRef] [PubMed]
26. Milani, N. The resistance of *Varroa jacobsoni* Oud. to acaricides. *Apidologie* **1999**, *30*, 229–234. [CrossRef]
27. Akyol, E.; Özkök, D. The use of organic acids for varroa (*Varroa destructor*) control. *Uludag Bee J.* **2005**, *5*, 167–174.
28. Aydın, L. Control of honey bee diseases and pets in autumn. *Uludag Bee J.* **2005**, *5*, 159–161.
29. Isman, M.B. Plant essential oils for pest and disease management. *Crop Prot.* **2000**, *19*, 603–608. [CrossRef]
30. Emsen, B.; Dodoloğlu, A.; Genç, F. Natural compounds for the control of *Varroa destructor* in honey bees (*Apis mellifera* L.) and an economic assessment of these products. *Mellifera* **2010**, *10*, 32–36.
31. Bahreini, R. Comparison of two methods of applying oxalic acid for control of varroa. *J. Apic. Res.* **2003**, *42*, 82–83. [CrossRef]
32. Hatjina, F.; Haristos, L. Indirect effects of oxalic acid administered by trickling method on honey bee brood. *J. Apic. Res.* **2005**, *44*, 172–174. [CrossRef]
33. Gregorc, A.; Škerl, M.I.S. Toxicological and immunohistochemical testing of honeybees after oxalic acid and rotenone treatments. *Apidologie* **2007**, *38*, 296–305. [CrossRef]
34. Hernández, R.M.; Pascual, M.H.; Pérez, J.; del Nozal Nalda, M.J.; Meana, A. Short term negative effect of oxalic acid in "*Apis mellifera* Iberiensis". *Span. J. Agric. Res.* **2007**, *5*, 474–480. [CrossRef]
35. Silva-Zacarin, E.C.; Gregorc, A.; de Moraes, R.L.S. In situ localization of heat-shock proteins and cell death labelling in the salivary gland of acaricide-treated honeybee larvae. *Apidologie* **2006**, *37*, 507–516. [CrossRef]
36. Underwood, R.; Currie, R. Use of formic acid to control varroa and tracheal mites in indoor overwintering facilities. *Am. Bee J.* **2003**, *143*, 323.
37. Higes, M.; Meana, A.; Suárez, M.; Llorente, J. Negative long-term effects on bee colonies treated with oxalic acid against *Varroa jacobsoni* Oud. *Apidologie* **1999**, *30*, 289–292. [CrossRef]

38. Borsuk, G.; Olszewski, K.; Paleolog, J.; Strachecka, A.; Gryzińska, M. The effect of different varroacides on the acidity of winter stores and honey stores. *Ann. UMCS Zootech.* **2012**, *30*, 11–16. [CrossRef]
39. Conti, B.; Bocchino, R.; Cosci, F.; Ascrizzi, R.; Flamini, G.; Bedini, S. Essential oils against *Varroa destructor*: A soft way to fight the parasitic mite of *Apis mellifera*. *J. Apic. Res.* **2020**, *59*, 774–782. [CrossRef]
40. Carson, C.F.; Hammer, K.A. Chemistry and bioactivity of essential oils. *Lipids Essent. Oils Antimicrob. Agents* **2011**, *25*, 203–238.
41. Athanassiou, C.G.; Rani, P.U.; Kavallieratos, N.G. The use of plant extracts for stored product protection. In *Advances in Plant Biopesticides*; Springer: Berlin/Heidelberg, Germany, 2014; pp. 131–147.
42. Bedini, S.; Flamini, G.; Girardi, J.; Cosci, F.; Conti, B. Not just for beer: Evaluation of spent hops (*Humulus lupulus* L.) as a source of eco-friendly repellents for insect pests of stored foods. *J. Pest Sci.* **2015**, *88*, 583–592. [CrossRef]
43. Bedini, S.; Cosci, F.; Tani, C.; Pierattini, E.C.; Venturi, F.; Lucchi, A.; Ioriatti, C.; Ascrizzi, R.; Flamini, G.; Ferroni, G. Essential oils as post-harvest crop protectants against the fruit fly *Drosophila suzukii*: Bioactivity and organoleptic profile. *Insects* **2020**, *11*, 508. [CrossRef]
44. Bedini, S.; Flamini, G.; Cosci, F.; Ascrizzi, R.; Echeverria, M.C.; Guidi, L.; Landi, M.; Lucchi, A.; Conti, B. *Artemisia* spp. essential oils against the disease-carrying blowfly *Calliphora vomitoria*. *Parasites Vectors* **2017**, *10*, 1–10. [CrossRef]
45. Omar, A.; Zayed, M. Bioactivity impact of essential oils *Allium sativum* L. and *Citrus reticulata* L. against stored product insects *Tribolium castaneum* (Herbst) and *Rhyzopertha dominica* (F.). *J. Plant Prot. Pathol.* **2021**, *12*, 465–471.
46. Ebadollahi, A.; Ziaee, M.; Palla, F. Essential oils extracted from different species of the lamiaceae plant family as prospective bioagents against several detrimental pests. *Molecules* **2020**, *25*, 1556. [CrossRef]
47. Bedini, S.; Bougherra, H.H.; Flamini, G.; Cosci, F.; Belhamel, K.; Ascrizzi, R.; Conti, B. Repellency of anethole-and estragole-type fennel essential oils against stored grain pests: The different twins. *Bull. Insectol.* **2016**, *69*, 149–157.
48. Conti, B.; Benelli, G.; Leonardi, M.; Afifi, F.U.; Cervelli, C.; Profeti, R.; Pistelli, L.; Canale, A. Repellent effect of *Salvia dorisiana*, *S. longifolia*, and *S. sclarea* (Lamiaceae) essential oils against the mosquito *Aedes albopictus* Skuse (Diptera: Culicidae). *Parasitol. Res.* **2012**, *111*, 291–299. [CrossRef] [PubMed]
49. Lima, T.C.; da Silva, T.K.M.; Silva, F.L.; Barbosa-Filho, J.M.; Marques, M.O.M.; Santos, R.L.C.; de Holanda Cavalcanti, S.C.; de Sousa, D.P. Larvicidal activity of *Mentha* x *villosa* Hudson essential oil, rotundifolone and derivatives. *Chemosphere* **2014**, *104*, 37–43. [CrossRef]
50. Benelli, G.; Bedini, S.; Cosci, F.; Toniolo, C.; Conti, B.; Nicoletti, M. Larvicidal and ovideterrent properties of neem oil and fractions against the filariasis vector *Aedes albopictus* (Diptera: Culicidae): A bioactivity survey across production sites. *Parasitol. Res.* **2015**, *114*, 227–236. [CrossRef] [PubMed]
51. Regnault-Roger, C.; Vincent, C.; Arnason, J.T. Essential oils in insect control: Low-risk products in a high-stakes world. *Annu. Rev. Èntomol.* **2012**, *57*, 405–424. [CrossRef]
52. Rajendran, S.; Sriranjini, V. Plant products as fumigants for stored-product insect control. *J. Stored Prod. Res.* **2008**, *44*, 126–135. [CrossRef]
53. Eguaras, M.J.; Fuselli, S.; Gende, L.; Fritz, R.; Ruffinengo, S.R.; Clemente, G.; Gonzalez, A.; Bailac, P.N.; Ponzi, M.I. An in vitro evaluation of *Tagetes minuta* essential oil for the control of the honeybee pathogens *Paenibacillus larvae* and *Ascosphaera apis*, and the parasitic mite *Varroa destructor*. *J. Essent. Oil Res.* **2005**, *17*, 336–340. [CrossRef]
54. Imdorf, A.; Bogdanov, S.; Ochoa, R.I.; Calderone, N.W. Use of essential oils for the control of *Varroa jacobsoni* Oud. in honey bee colonies. *Apidologie* **1999**, *30*, 209–228. [CrossRef]
55. Gashout, H.A.; Guzmán-Novoa, E. Acute toxicity of essential oils and other natural compounds to the parasitic mite, *Varroa destructor*, and to larval and adult worker honey bees (*Apis mellifera* L.). *J. Apic. Res.* **2009**, *48*, 263–269. [CrossRef]
56. Ramzi, H.; Ismaili, M.R.; Aberchane, M.; Zaanoun, S. Chemical characterization and acaricidal activity of *Thymus satureioides* C. & B. and *Origanum elongatum* E. & M. (*Lamiaceae)* essential oils against *Varroa destructor* Anderson & Trueman (Acari: Varroidae). *Ind. Crops Prod.* **2017**, *108*, 201–207.
57. Lindberg, C.M.; Melathopoulos, A.P.; Winston, M.L. Laboratory evaluation of miticides to csontrol *Varroa jacobsoni* (Acari: Varroidae), a honey bee (Hymenoptera: Apidae) parasite. *J. Econ. Entomol.* **2000**, *93*, 189–198. [CrossRef]
58. Adamczyk, S.; Lázaro, R.; Pérez-Arquillué, C.; Conchello, P.; Herrera, A. Evaluation of residues of essential oil components in honey after different anti-varroa treatments. *J. Agric. Food Chem.* **2005**, *53*, 10085–10090. [CrossRef] [PubMed]
59. Ariana, A.; Ebadi, R.; Tahmasebi, G. Laboratory evaluation of some plant essences to control *Varroa destructor* (Acari: Varroidae). *Exp. Appl. Acarol.* **2002**, *27*, 319–327. [CrossRef]
60. Aliano, N.P.; Ellis, M.D.; Siegfried, B.D. Acute contact toxicity of oxalic acid to *Varroa destructor* (Acari: Varroidae) and their *Apis mellifera* (Hymenoptera: Apidae) hosts in laboratory bioassays. *J. Econ. Entomol.* **2006**, *99*, 1579–1582. [CrossRef] [PubMed]
61. Ruffinengo, S.; Eguaras, M.; Floris, I.; Faverin, C.; Bailac, P.; Ponzi, M. LD50 and repellent effects of essential oils from Argentinian wild plant species on *Varroa destructor*. *J. Econ. Entomol.* **2005**, *98*, 651–655. [CrossRef] [PubMed]
62. Damiani, N.; Gende, L.B.; Bailac, P.; Marcangeli, J.A.; Eguaras, M.J. Acaricidal and insecticidal activity of essential oils on *Varroa destructor* (Acari: Varroidae) and *Apis mellifera* (Hymenoptera: Apidae). *Parasitol. Res.* **2009**, *106*, 145–152. [CrossRef] [PubMed]
63. Tihelka, E. Effects of synthetic and organic acaricides on honey bee health: A review. *Slov. Veter.-Res.* **2018**, *55*, 114–140. [CrossRef]
64. Charpentier, G.; Vidau, C.; Ferdy, J.; Tabart, J.; Vetillard, A. Lethal and sub-lethal effects of thymol on honeybee (*Apis mellifera*) larvae reared in vitro. *Pest Manag. Sci.* **2014**, *70*, 140–147. [CrossRef] [PubMed]

65. Boncristiani, H.; Underwood, R.; Schwarz, R.; Evans, J.D.; Pettis, J. Direct effect of acaricides on pathogen loads and gene expression levels in honey bees *Apis mellifera*. *J. Insect Physiol.* **2012**, *58*, 613–620. [CrossRef]
66. Bogdanov, S.; Imdorf, A.; Kilchenmann, V. Residues in wax and honey after Apilife VAR® treatment. *Apidologie* **1998**, *29*, 513–524. [CrossRef]
67. Floris, I.; Satta, A.; Cabras, P.; Garau, V.L.; Angioni, A. Comparison between two thymol formulations in the control of *Varroa destructor*: Effectiveness, persistence, and residues. *J. Econ. Entomol.* **2004**, *97*, 187–191. [CrossRef]
68. Tananaki, C.; Goras, G.; Huggett, N.; Karazafiris, E.; Dimou, M.; Thrasyvoulou, A. Evaluation of the impact of Exomite Pro™ on varroa mite (*Varroa destructor*) populations and honeybee (*Apis mellifera*) colonies: Efficacy, side effects and residues. *Parasitol. Res.* **2014**, *113*, 1251–1259. [CrossRef]
69. Ares, A.M.; Nozal, M.J.; Bernal, J.L.; Bernal, J. Simultaneous determination of carvacrol and thymol in bee pollen by using a simple and efficient solvent extraction method and gas chromatography-mass spectrometry. *J. Pharm. Biomed. Anal.* **2020**, *181*, 113124. [CrossRef]
70. Bergougnoux, M.; Treilhou, M.; Armengaud, C. Exposure to thymol decreased phototactic behaviour in the honeybee (*Apis mellifera*) in laboratory conditions. *Apidologie* **2013**, *44*, 82–89. [CrossRef]
71. Colin, T.; Lim, M.Y.; Quarrell, S.R.; Allen, G.R.; Barron, A.B. Effects of thymol on European honey bee hygienic behaviour. *Apidologie* **2019**, *50*, 141–152. [CrossRef]
72. Gunes, N.; Aydın, L.; Belenli, D.; Hranitz, J.M.; Mengilig, S.; Selova, S. Stress responses of honey bees to organic acid and essential oil treatments against varroa mites. *J. Apic. Res.* **2017**, *56*, 175–181. [CrossRef]
73. Jeong, E.-Y.; Jeon, J.-H.; Kim, H.-W.; Kim, M.-G.; Lee, H.-S. Antimicrobial activity of leptospermone and its derivatives against human intestinal bacteria. *Food Chem.* **2009**, *115*, 1401–1404. [CrossRef]
74. Jeong, E.; Kim, M.; Lee, H. Acaricidal activity of triketone analogues derived from *Leptospermum scoparium* oil against house-dust and stored-food mites. *Pest Manag. Sci.* **2009**, *65*, 327–331. [CrossRef]
75. Mattila, H.; Otis, G.; Daley, J.; Schulz, T. Trials of apiguard, a thymol-based miticide part 2. Non-target effects on honey bees. *Am. Bee J.* **2000**, *140*, 68–70.
76. Zambonelli, A.; D'Aulerio, A.Z.; Severi, A.; Benvenuti, S.; Maggi, L.; Bianchi, A. Chemical composition and fungicidal activity of commercial essential oils of *Thymus vulgaris* L. *J. Essent. Oil Res.* **2004**, *16*, 69–74. [CrossRef]
77. Nazer, I.; Al-Abbadi, A. Control of varroa mite (*Varroa destructor*) on honeybees by aromatic oils and plant materials. *J. Agric. Mar. Sci. [JAMS]* **2003**, *8*, 15–20. [CrossRef]
78. Thielmann, J.; Muranyi, P.; Kazman, P. Screening essential oils for their antimicrobial activities against the foodborne pathogenic bacteria *Escherichia coli* and *Staphylococcus aureus*. *Heliyon* **2019**, *5*, e01860. [CrossRef]
79. Ansari, M.J.; Al-Ghamdi, A.; Usmani, S.; Khan, K.A.; Alqarni, A.S.; Kaur, M.; Al-Waili, N. In vitro evaluation of the effects of some plant essential oils on *Ascosphaera apis*, the causative agent of chalkbrood disease. *Saudi J. Biol. Sci.* **2017**, *24*, 1001–1006. [CrossRef]
80. Ansari, M.J.; Al-Ghamdi, A.; Usmani, S.; Al-Waili, N.; Nuru, A.; Sharma, D.; Khan, K.A.; Kaur, M.; Omer, M. In vitro evaluation of the effects of some plant essential oils on *Paenibacillus larvae*, the causative agent of American foulbrood. *Biotechnol. Biotechnol. Equip.* **2016**, *30*, 49–55. [CrossRef]
81. Choi, Y.; Woo, S.; Hong, I.; Han, S.; Byoun, G.; Thapa, R.; Lee, M. Effects of limonene composition for controlling of the *Varroa destructor* and *Tropilaelaps clareae* in *Apis mellifera* Hives. *Korean J. Apic.* **2012**, *27*, 137–142.
82. van Vuuren, S.; Viljoen, A.M. Antimicrobial activity of limonene enantiomers and 1, 8-cineole alone and in combination. *Flavour Fragr. J.* **2007**, *22*, 540–544. [CrossRef]
83. Pattnaik, S.; Subramanyam, V.R.; Bapaji, M.; Kole, C.R. Antibacterial and antifungal activity of aromatic constituents of Essential oils. *Microbios* **1997**, *89*, 39–46.
84. Leite, A.M.; Lima, E.D.O.; De Souza, E.L.; Diniz, M.D.F.F.M.; Trajano, V.N.; De Medeiros, I.A. Inhibitory effect of beta-pinene, alpha-pinene and eugenol on the growth of potential infectious endocarditis causing gram-positive bacteria. *Rev. Bras. Ciênc. Farm.* **2007**, *43*, 121–126. [CrossRef]
85. Yang, J.; Nie, Q.; Ren, M.; Feng, H.; Jiang, X.; Zheng, Y.; Liu, M.; Zhang, H.; Xian, M. Metabolic engineering of *Escherichia coli* for the biosynthesis of alpha-pinene. *Biotechnol. Biofuels* **2013**, *6*, 1–10. [CrossRef] [PubMed]
86. Azevedo, M.; Chaves, F.; Almeida, C.A.; Bizzo, H.R.; Duarte, R.S.; Campos-Takaki, G.M.; Alviano, C.S.; Alviano, D.S. Antioxidant and antimicrobial activities of 7-hydroxy-calamenene-rich essential oils from *Croton cajucara* Benth. *Molecules* **2013**, *18*, 1128–1137. [CrossRef] [PubMed]
87. Liu, T.-T.; Yang, T.-S. Antimicrobial impact of the components of essential oil of *Litsea cubeba* from Taiwan and antimicrobial Activity of the oil in food systems. *Int. J. Food Microbiol.* **2012**, *156*, 68–75. [CrossRef]
88. Onawunmi, G.O. Evaluation of the antimicrobial activity of citral. *Lett. Appl. Microbiol.* **1989**, *9*, 105–108. [CrossRef]
89. Can Baser, K. Biological and pharmacological activities of carvacrol and carvacrol bearing essential oils. *Curr. Pharm. Des.* **2008**, *14*, 3106–3119. [CrossRef] [PubMed]
90. Doyle, A.A.; Stephens, J.C. A review of cinnamaldehyde and its derivatives as antibacterial agents. *Fitoterapia* **2019**, *139*, 104405. [CrossRef]

Effect of the digestive process of the Greater wax moth (*Galleria mellonella*) on the causative agents of American Foulbrood (*Paenibacillus larvae*)

Petr Mraz[1], Marian Hybl[3], Marek Kopecky[2], Jan Sipos[3], Stepan Ryba[4], Vladislav Curn[1]
[1]Department of Plant Production
[2]Department of Agroecosystems
University of South Bohemia in Ceske Budejovice
Studentska 1668, 370 05 Ceske Budejovice
[3]Department of Zoology, Fisheries, Hydrobiology and Apidology
Mendel University in Brno
Zemedelska 1, 613 00 Brno
[4]Biology Centre of Czech Academy of Sciences
Institute of Entomology
Branisovska 31, 370 05 Ceske Budejovice
CZECH REPUBLIC

mrazpe01@jcu.cz

Abstract: Both, greater wax moth (*Galleria mellonella*) and the causative agent of American Foulbrood (*Paenibacillus larvae*) cause considerable economic losses in beekeeping practice. Their life cycle is closely related to the honey bees, thus they can easily come into contact. The greater wax moth is exceptional for its ability to decompose beeswax. Therefore, it reduces also pathogen loads on old beeswax in nature. The aim of this study was to determine whether the greater wax moth is able to disrupt or destroy the very resistant spores of *P. larvae* in its digestive tract. In the laboratory experiment, the larvae of the greater wax moth were fed on wax foundation contaminated with *P. larvae* spores, either loosely or fixed in cages. After 2 days, their intestine was dissected and analysed by both, cultivation and molecular methods. The greatest amount of spores was found in the first parts of the intestine, fewer spores were found in the middle parts. No spores were detected in the back parts of the intestine. If the greater wax moth were able to destroy spores of the causative agent of American Foulbrood, there would be great potential for the development of a treatment that is still lacking. However, it is still not clear if the very efficient digestive tract of the greater wax moth is able to disrupt the spores or they are accumulated in the front part of the intestine.

Key Words: Honey bees, beekeeping pests, cultivation, digestive system, spores

INTRODUCTION

Pollinators, such as insects, birds, bats, and others, are very important for the sexual reproduction of many crops and wild plants. Bees are the main contributors to pollination and despite the great importance of solitary bees, the most numerous and also the most universal pollinator is the honey bee (*Apis mellifera*). Beekeeping, therefore, has a very close relationship with the environment and significantly contributes to strengthening the ecological stability of the landscape (Gallai et al. 2009). Honey bees are often the only way for farmers how to ensure sufficient pollination of crops if other pollinator species are rare or absent. Therefore, honey bees belong to one of the most important livestock (Klein et al. 2007, Morse and Calderon 2000).

However, despite the economic and environmental importance of bees, beekeeping is in decline throughout Europe (Biesmeijer et al. 2006). Increasing the application of agrochemicals to fields is a great risk, which significantly weakens bee colonies (Klein et al. 2017). Moreover, honey bee immunity is greatly influenced because of landscape changes, mainly manifested by malnutrition (Alaux et al. 2010, Hýbl et al. 2019). A combination of these negative factors makes bees more vulnerable to parasites and pathogens which resulted in a large decline of bees colonies every year.

One of the most economically serious diseases is American Foulbrood (AFB) which is caused by the bacteria *Paenibacillus larvae*. Once infected, the larva dies within 3 to 12 days. Adult bees cannot be infected, however, they can spread the disease (Genersch 2010). AFB spreads very easily both between hives and between localities (Lindström et al. 2008). The most common cause is the exchange of materials, bee robbing, or the sale of bee products that may be infected with this disease (Ashiralieva and Genersch 2006).

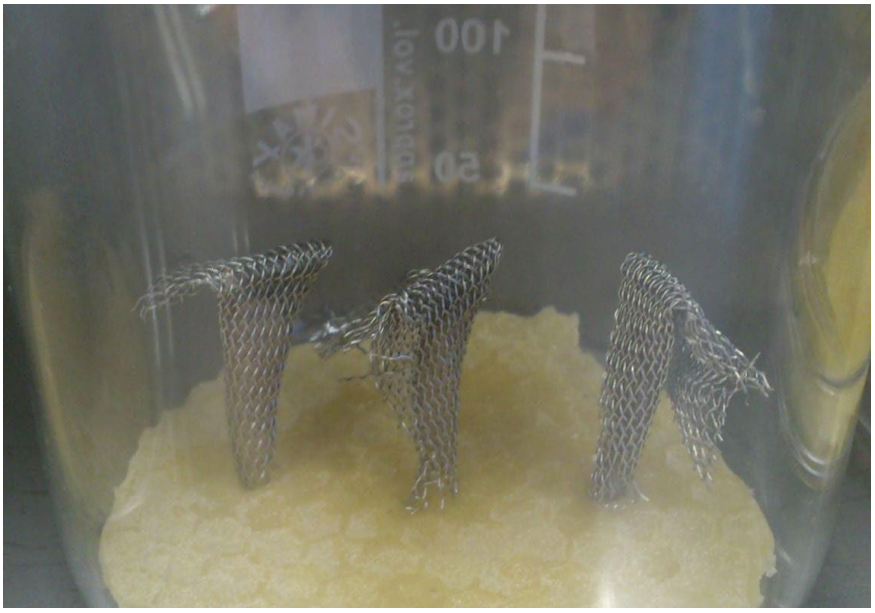
The greater wax moth is widespread and can be found in the beehive or on the old wax combs of the wild bees; therefore, it can easily come in contact with the bacteria causing AFB (Kwadha et al. 2017). It is considered a pest because its larvae feed mainly on older beeswax or combs with supplies of pollen. In nature, however, it plays an important role, namely the biological degradation of beeswax. It can even decompose very stable plastic materials (Billen et al. 2020, Bombelli et al. 2017), because their structures are similar to beeswax, both rich in long aliphatic chains (Kong et al. 2019). The process ensures the decomposition of very stable wax substances into simple components and their return back to the closed natural cycle. From this point of view, the larvae of the greater wax moth have a hygienic function in the landscape and also reduce the pathogen loads (Eillis et al. 2013). This raises the question of whether the larvae of the greater wax moth can deal with spores of *P. larvae*, which are commonly found in beeswax?

The aim of the work was to determine whether the greater wax moth is, thanks to its well-adapted digestive tract, able to disrupt the resistant layers of *P. larvae* spores and thus damage or destroy it.

MATERIAL AND METHODS

The larvae of greater wax moth (*Galleria mellonella*) were divided into 3 experimental groups. One of the test variants contained larvae, which were able to move freely inside the Petri dish (90 x 15 mm) on a wax foundation contaminated with spores of *P. larvae* (PK). For the second variant, small modified cages from mesh were prepared to prevent larval free movement (PR). The larvae were fixed inside the cages upside down and embedded in wax foundation contaminated with spores of *P. larvae* placed in a beaker (Figure 1). The fixed position ensured that the larvae were able to consume the contaminated wax but other parts of their body never came in contact with the spores. The spores had to go through the digestive system of larvae together with the food received. The last group consisted of larvae, which were able to move freely on the wax foundation without contamination (NK).

Figure 1 Larvae placed in cages



All of the larvae were fed for 2 days. Then, the larvae from the variants NK and PR were cut into 3 parts of the same size. The front part (1) consisted of a head and beginning of a digestive system.

The middle part (2) contained most of the digestive system and the back part (3) consisted of the end of digestive system and the Malpighian tubule system. From the last group of larvae (PK), the digestive system was dissected and divided into 3 parts in the same manner.

Spore extraction and inoculation

The sample amount of 0.1 g was diluted in 0.9 ml toluene and shaken for 1 hour. An amount of 0.2 ml of distilled water was added. The mixture was heated at 90 °C for 5 min to kill any undesirable bacteria. After cooling, 200 µl of the water phase was used for inoculation MYPGP agar and 100 µl of the water phase was used for the consequent PCR test (Ryba et al. 2009). All of the dishes were put in plastic bags to slightly increase CO_2 concentration and cultivated at 37 °C for 10 days. Growing colonies were assessed morphologically and identified by the peroxide test.

Molecular identification

The presence of spores was analysed also by PCR. Mixed samples from the PK and PR group were prepared. The whole DNA isolation procedure is according to the instructions of E.Z.N.A. Bacterial DNA Kit (2003). Genomic DNA of *P. larvae* was extracted and used as a template for PCR identification. The final volume was 50 µl including 1 µl of template DNA, 25 µl Master Mix, 20 µl of PCR Ultra H_2O and the specific ITS primers F3: TCCTGGCTCAGGACGAAC and B3: ACAGGTTGCCCCGTCTTT, 2 µl each at 10 µM. PCR was performed under the following conditions: initial template denaturation at 95 °C for 15 min; 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min. PCR products were electrophoretically separated on 1.0% agarose gel and visualized by a UV transilluminator (INGENIUS, Trigon-plus, SYNGENE) (Ryba et al. 2009).

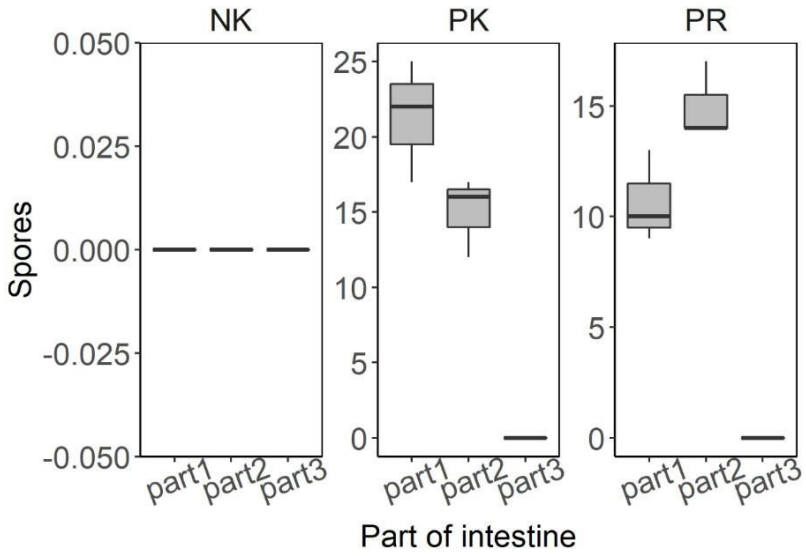
Statistical Analysis

The amount of viable spores between groups and part of intestine were statistically analysed by using repeated-measures nonparametric ANOVA (R Core Team 2020). Species groups and part of intestine were entered into the models as fixed effect, and each individual were entered as random effect. The post hoc comparison of the average of individual levels of fixed factors was compared by using the Tukey HSD test with Bonferroni corrected p-values.

RESULTS AND DISCUSSION

The number of viable spores in different experimental group and in each part of intestine differ significantly (ANOVA, $F_{(4,12)} = 59.323$, $p < 0.01$). As expected, the greater wax moth placed in an uncontaminated environment (NK) did not contain any *P. larvae* spores. The second variant which contained larvae placed in a contaminated environment (PK), was expected to have a high number of spores in all analysed parts. According to the cultivation tests, the front part contained a higher amount of spores. However, the middle part contained fewer spores (Tukey HSD, $p = 0.025$) and the back part did not even contain any spores. A slightly different situation occurred with the PR group. More spores were found in the middle part, less in the front part (Tukey HSD, $p = 0.019$) and the back part did not contain any spores (Figure 2). Overall, in the PK group, there were on average more than twice as many spores as in PR group in the front part of intestine. However, there were approximately equal numbers of viable spores in the middle intestines in both groups. That could be explained by the possible slow passage of spores through the digestive system of the greater wax moth. The spores are very small and can easily adhere to the surface of the intestine, which in addition has many folds (Kodrík 2004). The larvae in the PR group were fixed in small cages and could not move when they consumed all accessible diet. Therefore, their loads of spores were lower than in the PK group. In addition, more spores occurred in the middle part because many of the consumed spores passed through the front part. Due to the lack of accessible contaminated diet, no more spores were consumed. On the contrary, in the PK group, the intake of contaminated diet was unlimited and, therefore, more spores were accumulated in the front part. However, a very surprising result occurred in the back part where no spores were found. The absence of spores in the back part was confirmed also by the molecular analysis (Figure 3). Spores were either digested by the greater wax moth and intestinal microbiome enzymes (Cassone et al. 2020) or the passage through the intestine was very slow and spores were not able to reach the back part during the tested period of time.

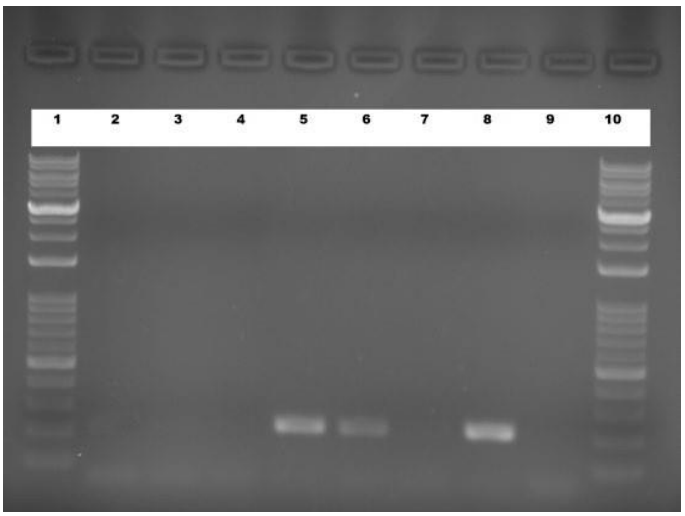
Figure 2 Number of colonies in cultivation test



Insect infection via the oral route is prevented both, by the structure of the gut which has a lining of chitin and by the adverse conditions in the gut such as pH and digestive enzymes. Furthermore, the intestinal microflora significantly contributes to the reduction of incoming microorganisms (Wojda 2017).

The other issue is *G. mellonella* immunity which could eliminate the pathogen. As same as other insect species, the greater wax moth has only innate immunity. However, if *G. mellonella* has previously encountered the pathogen in small amounts, its resistance increases (Bergin et al. 2006). This phenomenon is unusual in insects (Wojda 2017). However, this should be of no significance in this case, as the larvae were in contact with *P. larvae* for the first time. In addition, this bacterium is closely specialized for honey bee larvae and *G. mellonella* is not a suitable host (Genersch 2010).

Figure 3 Result of molecular analysis (PCR)



Legend: 1-Ladder 100bp, 2-NK first part of intestine, 3-NK second part of intestine, 4-NK third part of intestine, 5-PR+PK first part of intestine, 6- PR+PK second part of intestine, 7-PR+PK third part of intestine, 8-positive control, 9-negative control, 10-Ladder 100 bp

The greater wax moth immunity, development, and digestion efficiency is also influenced by the quality of food (Cassone et al. 2020, Kwadha et al. 2017). The natural diet is honey combs, honey, pollen and bee brood, but they can also be reared on artificial diets containing honey, wax and cereal products (Kwadha et al. 2017). In this case, the larvae were reared on artificial diet contained 660 g of corn bran, 330 g of wheat bran, 330 g of wheat flour, 330 g of powder milk, 165 g of dried yeast, 330 g of honey, 330 g of glycerine, 525 g of beeswax. Any negative side effect caused by the diet were not observed. Furthermore, an artificial diet can even support larval development and immunity

compared to natural diet (Jorjão et al. 2018). It is possible that naturally reared individuals can have different composition of microflora and thus the digestion efficiency between natural reared and artificial reared greater wax moth larvae can differ (Cassone et al. 2020).

CONCLUSIONS

The greater wax moth is known for its efficient digestive tract, which allows it to digest beeswax. At the same time, it significantly reduces the amount of bee pathogens, which occur mainly on old wax. On the other hand, spores of *P. larvae*, the causative agent of American Foulbrood, are very resistant. Therefore, the effect of the passage of *P. larvae* spores through the gastrointestinal tract of the greater wax moth was tested in this experiment. The main goal of the work was to determine the amount of spores and their germination after the passage. The results show that the most spores were in the front part of the intestine, the fewer spores were in the middle part and there were even no spores in the back part. However, it is not clear whether the spores in the digestive tract were digested or only accumulated in the anterior parts, which is full of folds and thus makes their passage more difficult. Therefore, further research is needed to clarify the details.

REFERENCES

Alaux, C. et al. 2010. Diet effects on honeybee immunocompetence. Biology Letters, 6(4): 562–565.

Ashiralieva, A., Genersch, E. 2006. Reclassifcation, genotypes and virulence of *Paenibacillus larvae*, the etiological agent of American foulbrood in honeybees - a review. Apidologie, 37(4): 411–420.

Bergin, D. et al. 2006. Pre-exposure to yeast protects larvae of *Galleria mellonella* from a subsequent lethal infection by *Candida albicans* and is mediated by the increased expression of antimicrobial peptides. Microbes Infect, 8: 2105–2112.

Biesmeijer, J.C. et al. 2006. Parallel declines in pollinators and insect pollinated plants in Britain and the Netherlands. Science, 313: 351–354.

Billen, P. et al. 2020. Technological application potential of polyethylene and polystyrene biodegradation by macro-organisms such as mealworms and wax moth larvae. Science of the Total Environment, 735: 1–10.

Bombelli, P. et al. 2017. Polyethylene bio-degradation by caterpillars of the wax moth *Galleria mellonella*. Current Biology, 27: 292–293.

Cassone, B.J. et al. 2020. Role of the intestinal microbiome in low-density polyethylene degradation by caterpillar larvae of the greater wax moth, *Galleria mellonella*. Proceedings of the Royal Society B. Biological Sciences, 287: 20200112.

Ellis, J.D. et al. 2013. Standard methods for wax moth research. Journal of Apicultural Research, 52(1): 1–17.

Gallai, N. et al. 2009. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecologicals Economics, 68: 810–821.

Genersch, E. 2010. American Foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. Journal of Invertebrate Pathology, 103: 10–19.

Hýbl, M. et al. 2019. Effects of phenolic bioactive substances on reducing mortality of bees (*Apis mellifera*) intoxicated by thiacloprid. In Proceedings of International PhD Students Conference MendelNet 2019 [Online]. Brno, Czech Republic, 6–7 November, Brno: Mendel University in Brno, Faculty of AgriSciences, pp. 131–135. Available at: https://mnet.mendelu.cz/mendelnet2019/mnet_2019_full.pdf

Jorjão, A.L. et al. 2018. From moths to caterpillars: Ideal conditions for *Galleria mellonella* rearing for in vivo microbiological studies. Virulence, 9(1): 383–389.

Klein, A.M. et al. 2007. Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society B. Biological Sciences, 274: 303–313.

Klein, S. et al. 2017. Why bees are so vulnerable to environmental stressors. Trends in Ecology and Evolution, 32(4): 268–278.

Kodrík, D. 2004. Fyziologie hmyzu, učební texty. České Budějovice: Jihočeská univerzita.

Kong, H.G. et al. 2019. The *Galleria mellonella* hologenome supports microbiota-independent metabolism of long-chain hydrocarbon beeswax. Cell Reports, 26: 2451–2464.

Kwadha, C.A. et al. 2017. The Biology and Control of the Greater Wax Moth, *Galleria mellonella*. Insects, 8(61): 1–17.

Lindström, A. et al. 2008. Horizontal transmission of *Paenibacillus larvae* spores between honey bee (*Apis mellifera*) colonies through robbing. Apidologie, 39: 515–522.

Morse, R.A., Calderon, N.W. 2000. The value of honey bee pollination in the United States. Bee Culture, 128: 1–15.

Ryba, S. et al. 2009. A PCR method of detecting American Foulbrood (*Paenibacillus larvae*) in winter beehive wax debris. Veterinary Microbiology, 139: 193–196.

6 Závěr

Disertační práce se zabývá hmyzími opylovateli a jejich významem a zároveň upozorňuje na jejich úbytek vlivem chemizace a intenzifikace zemědělství. Velká pozornost je věnována včele medonosné jako hlavnímu opylovateli, zejména schopnosti detoxikace pesticidů a alternativním možnostem kontroly vybraných patogenů. Disertační práce byla zaměřena do šesti tematických bloků a těmto blokům odpovídaly i cíle této práce. Naplnění všech cílů disertační práce je dokumentováno přiloženými publikovaným pracemi. V průběhu řešení byly i zodpovězeny vědecké hypotézy, jejich naplnění je uvedeno následovně:

6.1 Hodnocení vlivu a kvality opylení na kvantitativní a kvalitativní parametry výnosu zimolezu modrého

H: Opylování přirozenými opylovateli zvyšuje kvalitu a množství vyprodukovaných plodů.

- Tato hypotéza byla naplněna. Opylování přirozenými opylovateli zvyšuje kvalitu i množství vyprodukovaných plodů, a to jak v porovnání s umělým opylením, tak i variantou bez opylení.

6.2 Studium diverzity a početnosti včel v různých režimech hospodaření

H: Na lokalitě s ekologickým režimem hospodaření se vyskytuje více druhů včel s vyšší početností v porovnání s lokalitou s konvenčním hospodařením.

- Tato hypotéza byla naplněna. Na lokalitě s ekologickým režimem hospodaření se vyskytovalo významně více druhů včel a dosahovaly také výrazně vyšší početnosti, než na lokalitě s konvenčním režimem hospodaření.

H: Na lokalitě s ekologickým režimem hospodaření se vyskytují rezidua pesticidů v nižší míře než na lokalitě s konvenčním režimem hospodaření.

- Tato hypotéza byla naplněna. Na lokalitě s konvenčním režimem hospodaření byla ve včelách detekována rezidua několika pesticidů, zatímco na lokalitě s ekologickým režimem hospodaření nebyla detekována žádná.

6.3 Studium vlivu polyfenolů na schopnost detoxikace včel intoxikovaných pesticidem

H: Včely krmené stravou s přídavkem polyfenolů mají vyšší aktivitu detoxikačních enzymů.

- Tato hypotéza nebyla naplněna. Z výsledků není patrný nárůst exprese detoxikačních genů po podání fenolických látek. Důvodem může být delší časový interval mezi počátkem příjmu fenolických látek a samotným měření exprese detoxikačních genů.

H: Včely krmené stravou s přídavkem polyfenolů přežívají delší dobu.

- Tato hypotéza byla naplněna. Delší přežívání včel intoxikovaných pesticidem po podání fenolických látek bylo potvrzeno, stejně jako i vyšší míra spotřeby krmiva, což může signalizovat velký význam těchto látek ve výživě včel.

6.4 Hodnocení diverzity a početnosti vybraných včelích patogenů v různých typech habitatů

H: Z testovaných patogenů se v České republice vyskytuje nejčastěji *Nosema ceranae*.

- Tato hypotéza nebyla naplněna. Překvapivě byl nejčastěji detekován patogen *Lotmaria passim* patřící do čeledi Trypanosomatidae. Druhý nejčastěji detekovaný patogen již byla *Nosema ceranae*.

H: Typ ekosystému má vliv na výskyt včelích patogenů.

- Tato hypotéza byla naplněna jen částečně. Průkazně se potvrdil pouze výskyt nižšího počtu druhů patogenů v habitatu národního parku v porovnání s habitatem města. V zemědělsky intenzivně obhospodařované krajině již nebyl statisticky průkazný rozdíl. Výskyt virů také nebyl průkazně ovlivněn typem habitatu.

H: *Nosema apis* se v České republice vyskytuje velmi málo nebo vůbec.

- Tato hypotéza byla naplněna. Patogen *Nosema apis* nebyl detekován v žádném ze vzorků.

H: Patogeny včel ovlivňují přezimování včelstev.

- Tato hypotéza byla naplněna. Průkazně však byl prokázán negativní vliv na přezimování včelstev pouze u virových patogenů, konkrétně DWV – B a DWV – C.

6.5 Testování nových a alternativních možností kontroly vybraných včelích patogenů pomocí rostlinných silic

H: Včelí plod má pozitivní vliv na růst a produkci reprodukčních struktur entomopatogenní houby *A. apis*.

- Tato hypotéza byla naplněna. Vysoce pozitivní vliv byl zaznamenán zejména v produkci reprodukčních struktur. Pozitivní vliv byl pozorován i v radiálním růstu, ale některá kultivační media byla v tomto ohledu ještě vhodnější.

H: Některé z vybraných rostlinných silic mají fungicidní nebo akaricidní efekt.

- Tato hypotéza byla naplněna. U velkého počtu testovaných rostlinných silic byl potvrzen značný fungicidní nebo akaricidní efekt. U některých silic dokonce i jejich kombinace.

H: Některé z vybraných rostlinných silic mají větší toxický efekt na roztoče než na včely.

- Tato hypotéza byla potvrzena. Všechny z testovaných nejvíce účinných rostlinných silic měly větší toxický efekt na roztoče než na včely. Některé dokonce mnohanásobně.

H: Využití rostlinných silic ve včelařské praxi je teoreticky možné.

- Tato hypotéza byla potvrzena. Vzhledem k vysoké toxicitě některých rostlinných silic na roztoče *Varroa* a zároveň značné toleranci včel je teoreticky možné jejich využití ve včelařské praxi. Bude však nezbytné provést další experimenty, optimálně v poloprovozních podmínkách a stanovit vhodnou aplikační formu i dávku silic.

6.6 Studium nových a alternativních možností kontroly vybraných včelích patogenů pomocí trávicích enzymů zavíječe voskového

H: Trávicí systém zavíječe voskového narušuje spory původce moru včelího plodu.

- Tato hypotéza nebyla jednoznačně potvrzena ani vyvrácena. Spory bakterie *P. larvae* sice nebyly nalezeny v zadní části trávicího traktu zavíječe voskového, ale nebylo prokázáno ani jejich narušení. Spory se totiž mohly akumulovat v předních částech trávicího traktu. Pro objasnění by bylo vhodné provést další experimenty.

7 Seznam použité literatury

Abd El-Wahab, T.E., Ebadah, I.M.A., Zidan, E.W., 2012. Control of Varroa mite by essential oils and formic acid with their effects on grooming behaviour of honey bee colonies. J. Basic. Appl. Sci. Res 2, 7674–7680.

Aglagane, A., Laghzaoui, E.-M., Soulaimani, B., Er-Rguibi, O., Abbad, A., Mouden, E.H.E., Aourir, M., 2021. Acaricidal activity of Mentha suaveolens subsp. timija, Chenopodium ambrosioides, and Laurus nobilis essential oils, and their synergistic combinations against the ectoparasitic bee mite, Varroa destructor (Acari: Varroidae). Journal of Apicultural Research 1–10.

Aizen, M.A., Garibaldi, L.A., Cunningham, S.A., Klein, A.M., 2009. How much does agriculture depend on pollinators? Lessons from long-term trends in crop production. Annals of botany 103, 1579–1588.

Al Toufailia, H., Scandian, L., Ratnieks, F.L., 2015. Towards integrated control of varroa: 2) comparing application methods and doses of oxalic acid on the mortality of phoretic Varroa destructor mites and their honey bee hosts. Journal of Apicultural Research 54, 108–120.

Alghamdi, A., Dalton, L., Phillis, A., Rosato, E., Mallon, E.B., 2008. Immune response impairs learning in free-flying bumble-bees. Biology Letters 4, 479–481.

Aliano, N.P., Ellis, M.D., 2005. A strategy for using powdered sugar to reduce varroa populations in honey bee colonies. Journal of Apicultural Research 44, 54–57.

Aliano, N.P., Ellis, M.D., Siegfried, B.D., 2006. Acute contact toxicity of oxalic acid to Varroa destructor (Acari: Varroidae) and their Apis mellifera (Hymenoptera: Apidae) hosts in laboratory bioassays. Journal of economic Entomology 99, 1579–1582.

Alotaibi, A., Ebiloma, G.U., Williams, R., Alenezi, S., Donachie, A.-M., Guillaume, S., Igoli, J.O., Fearnley, J., De Koning, H.P., Watson, D.G., 2019. European propolis is highly active against trypanosomatids including Crithidia fasciculata. Scientific reports 9, 1–10.

Al-Waili, N., Salom, K., Al-Ghamdi, A., Ansari, M.J., 2012. Antibiotic, pesticide, and microbial contaminants of honey: human health hazards. The scientific world Journal 2012.

Amdam, G.V., Hartfelder, K., Norberg, K., Hagen, A., Omholt, S.W., 2004. Altered physiology in worker honey bees (Hymenoptera: Apidae) infested with the mite Varroa destructor (Acari: Varroidae): a factor in colony loss during overwintering? Journal of economic entomology 97, 741–747.

Anderson, D., Trueman, J., 2000. Varroa jacobsoni (acari: varroidae) is more than one species. Experimental & applied acarology 24, 165–189.

Angioni, A., Barra, A., Coroneo, V., Dessi, S., Cabras, P., 2006. Chemical composition, seasonal variability, and antifungal activity of Lavandula stoechas L. ssp. stoechas essential oils from stem/leaves and flowers. Journal of agricultural and food chemistry 54, 4364–4370.

Annoscia, D., Di Prisco, G., Becchimanzi, A., Caprio, E., Frizzera, D., Linguadoca, A., Nazzi, F., Pennacchio, F., 2020. Neonicotinoid Clothianidin reduces honey bee immune response and contributes to Varroa mite proliferation. Nature communications 11, 1–7.

Ansari, M.J., Al-Ghamdi, A., Usmani, S., Khan, K.A., Alqarni, A.S., Kaur, M., Al-Waili, N., 2017. In vitro evaluation of the effects of some plant essential oils on Ascosphaera apis, the causative agent of Chalkbrood disease. Saudi journal of biological sciences 24, 1001–1006.

Antúnez, K., Anido, M., Schlapp, G., Evans, J.D., Zunino, P., 2009. Characterization of secreted proteases of Paenibacillus larvae, potential virulence factors involved in honeybee larval infection. Journal of invertebrate pathology 102, 129–132.

Ariana, A., Ebadi, R., Tahmasebi, G., 2002. Laboratory evaluation of some plant essences to control Varroa destructor (Acari: Varroidae). Experimental & applied acarology 27, 319–327.

Arismendi, N., Vargas, M., López, M.D., Barría, Y., Zapata, N., 2018. Promising antimicrobial activity against the honey bee parasite Nosema ceranae by methanolic extracts from Chilean native plants and propolis. Journal of Apicultural Research 57, 522–535.

Aronstein, K.A., Murray, K.D., 2010. Chalkbrood disease in honey bees. Journal of invertebrate pathology 103, S20–S29.

Ash, C., Priest, F.G., Collins, M.D., 1993. Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Antonie van leeuwenhoek 64, 253–260.

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils–a review. Food and chemical toxicology 46, 446–475.

Bamford, S., Heath, L., 1989. The effects of temperature and pH on the germination of spores of the chalkbrood fungus, Ascosphaera apis. Journal of Apicultural Research 28, 36–40.

Baracchi, D., Fadda, A., Turillazzi, S., 2012. Evidence for antiseptic behaviour towards sick adult bees in honey bee colonies. Journal of insect physiology 58, 1589–1596.

Bargańska, Ż., Namieśnik, J., Ślebioda, M., 2011. Determination of antibiotic residues in honey. TrAC Trends in Analytical Chemistry 30, 1035–1041.

Berry, J.A., Afik, O., Nolan IV, M.P., Delaplane, K.S., 2012. Revisiting powdered sugar for varroa control on honey bees (Apis mellifera L.). Journal of Apicultural Research 51, 367–368.

Berry, J.A., Owens, W.B., Delaplane, K.S., 2010. Small-cell comb foundation does not impede Varroa mite population growth in honey bee colonies. Apidologie 41, 40–44.

Bevk, D., Kralj, J., Čokl, A., 2012. Coumaphos affects food transfer between workers of honeybee Apis mellifera. Apidologie 43, 465–470.

Biesmeijer, J.C., Roberts, S.P., Reemer, M., Ohlemüller, R., Edwards, M., Peeters, T., Schaffers, A.P., Potts, S.G., Kleukers, R., Thomas, C.D., 2006. Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. Science 313, 351–354.

Bíliková, K., Wu, G., Šimúth, J., 2001. Isolation of a peptide fraction from honeybee royal jelly as a potential antifoulbrood factor. Apidologie 32, 275–283.

Billen, P., Khalifa, L., Van Gerven, F., Tavernier, S., Spatari, S., 2020. Technological application potential of polyethylene and polystyrene biodegradation by macro-organisms such as mealworms and wax moth larvae. Science of the Total Environment 735, 139521.

Bolatovna, K.S., Rustenov, A., Eleuqalieva, N., Omirzak, T., Akhanov, U.K., 2015. Improving reproductive qualities of pigs using the drone brood homogenate. Biol Med (Aligarh) 7, 2.

Bombelli, P., Howe, C.J., Bertocchini, F., 2017. Polyethylene bio-degradation by caterpillars of the wax moth Galleria mellonella. Current biology 27, R292–R293.

Borba, R.S., Spivak, M., 2017. Propolis envelope in Apis mellifera colonies supports honey bees against the pathogen, Paenibacillus larvae. Scientific reports 7, 1–6.

Borges, D., Guzman-Novoa, E., Goodwin, P.H., 2020. Control of the microsporidian parasite Nosema ceranae in honey bees (Apis mellifera) using nutraceutical and immuno-stimulatory compounds. Plos one 15, e0227484.

Branco, M.R., Kidd, N.A., Pickard, R.S., 2006. A comparative evaluation of sampling methods for Varroa destructor (Acari: Varroidae) population estimation. Apidologie 37, 452–461.

Brasesco, M.C., Gende, L.B., Negri, P., Szawarski, N., Iglesias, A.E., Eguaras, M.J., Ruffinengo, S.R., Maggi, M.D., 2017. Assessing in vitro acaricidal effect and joint action of a binary mixture between essential oil compounds (thymol, phellandrene, eucalyptol, cinnamaldehyde, myrcene, carvacrol) over ectoparasitic mite Varroa destructor (Acari: Varroidae).

Bravo, J., Carbonell, V., Sepúlveda, B., Delporte, C., Valdovinos, C., Martín-Hernández, R., Higes, M., 2017. Antifungal activity of the essential oil obtained from Cryptocarya alba against infection in honey bees by Nosema ceranae. Journal of invertebrate pathology 149, 141–147.

Brodschneider, R., Brus, J., Danihlík, J., 2019. Comparison of apiculture and winter mortality of honey bee colonies (Apis mellifera) in Austria and Czechia. Agriculture, Ecosystems & Environment 274, 24–32.

Burley, L.M., Fell, R.D., Saacke, R.G., 2008. Survival of honey bee (Hymenoptera: Apidae) spermatozoa incubated at room temperature from drones exposed to miticides. Journal of economic entomology 101, 1081–1087.

Calderone, N., 2005. Evaluation of drone brood removal for management of Varroa destructor (Acari: Varroidae) in colonies of Apis mellifera (Hymenoptera: Apidae) in the northeastern United States. Journal of Economic Entomology 98, 645–650.

Calderone, N.W., Shimanuki, H., Allen-Wardell, G., 1994. An in vitro evaluation of botanical compounds for the control of the honeybee pathogens Bacillus larvae and Ascosphaera apis, and the secondary invader B. alvei. Journal of Essential Oil Research 6, 279–287.

Campbell, E.M., Budge, G.E., Watkins, M., Bowman, A.S., 2016. Transcriptome analysis of the synganglion from the honey bee mite, Varroa destructor and RNAi knockdown of neural peptide targets. Insect biochemistry and molecular biology 70, 116–126.

Cengiz, M.M., 2018. Effectiveness of combining certain biotechnical methods with thymol treatment against Varroa destructor infestation. African Journal of Agricultural Research 13, 2735–2740.

Chaimanee, V., Thongtue, U., Sornmai, N., Songsri, S., Pettis, J.S., 2017. Antimicrobial activity of plant extracts against the honeybee pathogens, Paenibacillus larvae and Ascosphaera apis and their topical toxicity to Apis mellifera adults. Journal of applied microbiology 123, 1160–1167.

Chantawannakul, P., Puchanichanthranon, T., Wongsiri, S., 2003. Inhibitory effects of some medicinal plant extracts on the growth of Ascosphaera apis, in: III WOCMAP Congress on Medicinal and Aromatic Plants-Volume 4: Targeted Screening of Medicinal and Aromatic Plants, Economics 678. pp. 183–189.

Charriére, J.-D., Imdorf, A., 2002. Oxalic acid treatment by trickling against Varroa destructor: recommendations for use in central Europe and under temperate climate conditions. Bee world 83, 51–60.

Chautá-Mellizo, A., Campbell, S.A., Bonilla, M.A., Thaler, J.S., Poveda, K., 2012. Effects of natural and artificial pollination on fruit and offspring quality. Basic and Applied Ecology 13, 524–532.

Chen, Y.P., Pettis, J.S., Corona, M., Chen, W.P., Li, C.J., Spivak, M., Visscher, P.K., DeGrandi-Hoffman, G., Boncristiani, H., Zhao, Y., 2014. Israeli acute paralysis virus: epidemiology, pathogenesis and implications for honey bee health. PLoS Pathog 10, e1004261.

Cianciosi, D., Forbes-Hernández, T.Y., Afrin, S., Gasparrini, M., Reboredo-Rodriguez, P., Manna, P.P., Zhang, J., Bravo Lamas, L., Martínez Flórez, S., Agudo Toyos, P., 2018. Phenolic compounds in honey and their associated health benefits: A review. Molecules 23, 2322.

Colin, T., Lim, M.Y., Quarrell, S.R., Allen, G.R., Barron, A.B., 2019. Effects of thymol on European honey bee hygienic behaviour. Apidologie 50, 141–152.

Collins, A.M., Pettis, J.S., 2013. Correlation of queen size and spermathecal contents and effects of miticide exposure during development. Apidologie 44, 351–356.

Conti, B., Bocchino, R., Cosci, F., Ascrizzi, R., Flamini, G., Bedini, S., 2020. Essential oils against Varroa destructor: a soft way to fight the parasitic mite of Apis mellifera. Journal of Apicultural Research 59, 774–782.

Cooper, E.L., 2006. Comparative immunology. Integrative zoology 1, 32–43.

Cornara, L., Biagi, M., Xiao, J., Burlando, B., 2017. Therapeutic properties of bioactive compounds from different honeybee products. Frontiers in pharmacology 8, 412.

Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D., Moran, N.A., Quan, P.-L., Briese, T., Hornig, M., Geiser, D.M., 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science 318, 283–287.

Damiani, N., Fernández, N.J., Porrini, M.P., Gende, L.B., Álvarez, E., Buffa, F., Brasesco, C., Maggi, M.D., Marcangeli, J.A., Eguaras, M.J., 2014. Laurel leaf extracts for honeybee pest and disease management: antimicrobial, microsporicidal, and acaricidal activity. Parasitology research 113, 701–709.

Damiani, N., Gende, L.B., Bailac, P., Marcangeli, J.A., Eguaras, M.J., 2009. Acaricidal and insecticidal activity of essential oils on Varroa destructor (Acari: Varroidae) and Apis mellifera (Hymenoptera: Apidae). Parasitology research 106, 145–152.

de Mattos, I.M., Soares, A.E., Tarpy, D.R., 2017. Effects of synthetic acaricides on honey bee grooming behavior against the parasitic Varroa destructor mite. Apidologie 48, 483–494.

Desai, S.D., Currie, R.W., 2015. Genetic diversity within honey bee colonies affects pathogen load and relative virus levels in honey bees, Apis mellifera L. Behavioral ecology and sociobiology 69, 1527–1541.

Di Prisco, G., Annoscia, D., Margiotta, M., Ferrara, R., Varricchio, P., Zanni, V., Caprio, E., Nazzi, F., Pennacchio, F., 2016. A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health. Proceedings of the National Academy of Sciences 113, 3203–3208.

Dietemann, V., Ellis, J.D., Neumann, P., 2013. The COLOSS BEEBOOK Volume II: Standard methods for Apis mellifera pest and pathogen research. International Bee Research Association IBRA.

Dolezal, A.G., Clair, A.L.S., Zhang, G., Toth, A.L., O'Neal, M.E., 2019. Native habitat mitigates feast–famine conditions faced by honey bees in an agricultural landscape. Proceedings of the National Academy of Sciences 116, 25147–25155.

Domatskaya, T., Domatsky, A., 2020. Study of effectiveness of lactic acid at varroatosis in the apiaries of Tyumen region, Russia. Ukrainian Journal of Ecology 10, 155–159.

Duay, P., De Jong, D., Engels, W., 2003. Weight loss in drone pupae (Apis mellifera) multiply infested by Varroa destructor mites. Apidologie 34, 61–65.

Duay, P., De Jong, D., Engels, W., 2002. Decreased flight performance and sperm production in drones of the honey bee (Apis mellifera) slightly infested by Varroa destructor mites during pupal development. Genet. Mol. Res 1, 227–232.

Dubovskiy, I., Kryukova, N., Glupov, V., Ratcliffe, N., 2016. Encapsulation and nodulation in insects. Invertebrate Survival Journal 13, 229–246.

Ebert, T.A., Kevan, P.G., Bishop, B.L., Kevan, S.D., Downer, R.A., 2007. Oral toxicity of essential oils and organic acids fed to honey bees (Apis mellifera). Journal of apicultural research 46, 220–224.

Eilers, E.J., Kremen, C., Greenleaf, S.S., Garber, A.K., Klein, A.-M., 2011. Contribution of pollinator-mediated crops to nutrients in the human food supply. PLoS one 6, e21363.

Ellis, A., Hayes, G., Ellis, J., 2009. The efficacy of small cell foundation as a varroa mite (Varroa destructor) control. Experimental and Applied Acarology 47, 311–316.

Ellis, A.M., Hayes, G.W., Ellis, J.D., 2009. The efficacy of dusting honey bee colonies with powdered sugar to reduce Varroa mite populations. Journal of apicultural research 48, 72–76.

Ellis, J.D., Graham, J.R., Mortensen, A., 2013. Standard methods for wax moth research. Journal of Apicultural Research 52, 1–17.

Evans, J., Aronstein, K., Chen, Y.P., Hetru, C., Imler, J., Jiang, H., Kanost, M., Thompson, G., Zou, Z., Hultmark, D., 2006. Immune pathways and defence mechanisms in honey bees Apis mellifera. Insect molecular biology 15, 645–656.

Evans, J.D., Armstrong, T.-N., 2005. Inhibition of the American foulbrood bacterium, Paenibacillus larvae larvae, by bacteria isolated from honey bees. Journal of Apicultural Research 44, 168–171.

Evans, J.D., Spivak, M., 2010. Socialized medicine: individual and communal disease barriers in honey bees. Journal of invertebrate pathology 103, S62–S72.

Evison, S.E., 2015. Chalkbrood: epidemiological perspectives from the host–parasite relationship. Current opinion in insect science 10, 65–70.

Fakhimzadeh, K., 2001a. Effectiveness of confectioner sugar dusting to knock down Varroa destructor from adult honey bees in laboratory trials. Apidologie 32, 139–148.

Fakhimzadeh, K., 2001b. The effects of powdered sugar varroa control treatments on Apis mellifera colony development. Journal of Apicultural Research 40, 105–109.

Ferrentino, G., Morozova, K., Horn, C., Scampicchio, M., 2020. Extraction of essential oils from medicinal plants and their utilization as food antioxidants. Current pharmaceutical design 26, 519–541.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. nature 391, 806–811.

Flenniken, M.L., Andino, R., 2013. Non-specific dsRNA-mediated antiviral response in the honey bee. Plos one 8, e77263.

Flores, J., Spivak, M., Gutiérrez, I., 2005. Spores of Ascosphaera apis contained in wax foundation can infect honeybee brood. Veterinary Microbiology 108, 141–144.

Frazier, M., Mullin, C., Frazier, J., Ashcraft, S., 2008. What have pesticides got to do with it? American Bee Journal 148, 521–524.

Fries, I., Aarhus, A., Hansen, H., Korpela, S., 1991. Comparison of diagnostic methods for detection of low infestation levels of Varroa jacobsoni in honey-bee (Apis mellifera) colonies. Experimental & applied acarology 10, 279–287.

Gabriel, K.T., Kartforosh, L., Crow, S.A., Cornelison, C.T., 2018. Antimicrobial activity of essential oils against the fungal pathogens Ascosphaera apis and Pseudogymnoascus destructans. Mycopathologia 183, 921–934.

Gal, H., Slabezki, Y., Lensky, Y., 1992. A preliminary report on the effect of origanum oil and thymol applications in honey bee (Apis mellifera L.) colonies in a subtropical climate on population levels of Varroa jacobsoni. Bee Sci 2, 175–180.

Gallai, N., Salles, J.-M., Settele, J., Vaissière, B.E., 2009. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecological economics 68, 810–821.

Garbian, Y., Maori, E., Kalev, H., Shafir, S., Sela, I., 2012. Bidirectional transfer of RNAi between honey bee and Varroa destructor: Varroa gene silencing reduces Varroa population. PLoS Pathog 8, e1003035.

García-Fernández, P., Santiago-Álvarez, C., Quesada-Moraga, E., 2008. Pathogenicity and thermal biology of mitosporic fungi as potential microbial control agents of Varroa destructor (Acari: Mesostigmata), an ectoparasitic mite of honey bee, Apis mellifera (Hymenoptera: Apidae). Apidologie 39, 662–673.

Gashout, H.A., Goodwin, P.H., Guzman-Novoa, E., 2018. Lethality of synthetic and natural acaricides to worker honey bees (Apis mellifera) and their impact on the expression

of health and detoxification-related genes. Environmental Science and Pollution Research 25, 34730–34739.
Gashout, H.A., Guzman-Novoa, E., Goodwin, P.H., Correa-Benítez, A., 2020. Impact of sublethal exposure to synthetic and natural acaricides on honey bee (Apis mellifera) memory and expression of genes related to memory. Journal of insect physiology 121, 104014.
Genersch, E., 2010a. Honey bee pathology: current threats to honey bees and beekeeping. Applied microbiology and biotechnology 87, 87–97.
Genersch, E., 2010b. American Foulbrood in honeybees and its causative agent, Paenibacillus larvae. Journal of invertebrate pathology 103, S10–S19.
Genersch, E., Ashiralieva, A., Fries, I., 2005. Strain-and genotype-specific differences in virulence of Paenibacillus larvae subsp. larvae, a bacterial pathogen causing American foulbrood disease in honeybees. Applied and Environmental Microbiology 71, 7551–7555.
Genersch, E., Forsgren, E., Pentikäinen, J., Ashiralieva, A., Rauch, S., Kilwinski, J., Fries, I., 2006. Reclassification of Paenibacillus larvae subsp. pulvifaciens and Paenibacillus larvae subsp. larvae as Paenibacillus larvae without subspecies differentiation. International Journal of Systematic and Evolutionary Microbiology 56, 501–511.
Girişgin, A., Aydin, L., 2010. Efficacies of formic, oxalic and lactic acids against Varroa destructor in naturally infested honeybee (Apis mellifera L.) colonies in Turkey. Kafkas Univ Vet Fak Derg 16, 941–945.
Gisder, S., Aumeier, P., Genersch, E., 2009. Deformed wing virus: replication and viral load in mites (Varroa destructor). Journal of General Virology 90, 463–467.
Gisder, S., Genersch, E., 2021. Direct Evidence for Infection of Varroa destructor Mites with the Bee-Pathogenic Deformed Wing Virus Variant B, but Not Variant A, via Fluorescence In Situ Hybridization Analysis. Journal of Virology 95, e01786-20.
Gisder, S., Möckel, N., Eisenhardt, D., Genersch, E., 2018. In vivo evolution of viral virulence: switching of deformed wing virus between hosts results in virulence changes and sequence shifts. Environmental microbiology 20, 4612–4628.
Giusti, M., Sabelli, C., Di Donato, A., Lamberti, D., Paturzo, C.E., Polignano, V., Lazzari, R., Felicioli, A., 2017. Efficacy and safety of Varterminator, a new formic acid medicine against the varroa mite. Journal of apicultural research 56, 162–167.
Glavan, G., Novak, S., Božič, J., Kokalj, A.J., 2020. Comparison of sublethal effects of natural acaricides carvacrol and thymol on honeybees. Pesticide Biochemistry and Physiology 166, 104567.
Goblirsch, M., Warner, J.F., Sommerfeldt, B.A., Spivak, M., 2020. Social fever or general immune response? Revisiting an example of social immunity in honey bees. Insects 11, 528.
Gómez-Moracho, T., Heeb, P., Lihoreau, M., 2017. Effects of parasites and pathogens on bee cognition. Ecological Entomology 42, 51–64.
Gonzalez-Cabrera, J., Rodriguez-Vargas, S., Davies, T.E., Field, L.M., Schmehl, D., Ellis, J.D., Krieger, K., Williamson, M.S., 2016. Novel mutations in the voltage-gated sodium channel of pyrethroid-resistant Varroa destructor populations from the Southeastern USA. PloS one 11, e0155332.
Goodwin, R.M., Perry, J.H., Houten, A.T., 1994. The effect of drifting honey bees on the spread of American foulbrood infections. Journal of Apicultural Research 33, 209–212.
Goulson, D., Nicholls, E., Botías, C., Rotheray, E.L., 2015. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science 347.
Gregorc, A., Knight, P.R., Adamczyk, J., 2017. Powdered sugar shake to monitor and oxalic acid treatments to control varroa mites (Varroa destructor Anderson and Trueman) in honey bee (Apis mellifera) colonies. Journal of Apicultural Research 56, 71–75.

Gregorc, A., Planinc, I., 2012. Use of thymol formulations, amitraz, and oxalic acid for the control of the varroa mite in honey bee (Apis mellifera carnica) colonies. Apicultural Science 56, 61.

Gregorc, A., Planinc, I., 2001. Acaricidal effect of oxalic acid in honeybee (Apis mellifera) colonies. Apidologie 32, 333–340.

Gregorc, A., Sampson, B., 2019. Diagnosis of Varroa Mite (Varroa destructor) and sustainable control in honey bee (Apis mellifera) colonies—A review. Diversity 11, 243.

Gregorc, A., Škerl, M.I.S., 2007. Toxicological and immunohistochemical testing of honeybees after oxalic acid and rotenone treatments. Apidologie 38, 296–305.

Guichard, M., Dietemann, V., Neuditschko, M., Dainat, B., 2020. Three decades of selecting honey bees that survive infestations by the parasitic mite Varroa destructor: outcomes, limitations and strategy.

Guo, R., Chen, D., Xiong, C., Hou, C., Zheng, Y., Fu, Z., Diao, Q., Zhang, L., Wang, H., Hou, Z., 2018. Identification of long non-coding RNAs in the chalkbrood disease pathogen Ascospheara apis. Journal of invertebrate pathology 156, 1–5.

Haber, A.I., Steinhauer, N.A., vanEngelsdorp, D., 2019. Use of chemical and nonchemical methods for the control of Varroa destructor (Acari: Varroidae) and associated winter colony losses in US beekeeping operations. Journal of economic entomology 112, 1509–1525.

Hallmann, C.A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Müller, A., Sumser, H., Hörren, T., 2017. More than 75 percent decline over 27 years in total flying insect biomass in protected areas. PloS one 12, e0185809.

Hansen, H., Brødsgaard, C.J., 1999. American foulbrood: a review of its biology, diagnosis and control. Bee world 80, 5–23.

Häußermann, C.K., Giacobino, A., Munz, R., Ziegelmann, B., Palacio, M.A., Rosenkranz, P., 2019. Reproductive parameters of female Varroa destructor and the impact of mating in worker brood of Apis mellifera. Apidologie 1–14.

Heath, L., Gaze, B.M., 1987. Carbon dioxide activation of spores of the chalkbrood fungus Ascosphaera apis. Journal of Apicultural Research 26, 243–246.

Hedtke, K., Jensen, P.M., Jensen, A.B., Genersch, E., 2011. Evidence for emerging parasites and pathogens influencing outbreaks of stress-related diseases like chalkbrood. Journal of invertebrate pathology 108, 167–173.

Hernández, R.M., Pascual, M.H., Pérez, J., del Nozal Nalda, M.J., Meana, A., 2007. Short term negative effect of oxalic acid in" Apis mellifera iberiensis". Spanish journal of agricultural research 5, 474–480.

Hernández-Rodríguez, C.S., Marín, Ó., Calatayud, F., Mahiques, M.J., Mompó, A., Segura, I., Simó, E., González-Cabrera, J., 2021. Large-Scale Monitoring of Resistance to Coumaphos, Amitraz, and Pyrethroids in Varroa destructor. Insects 12, 27.

Heyndrickx, M., Vandemeulebroecke, K., Hoste, B., Janssen, P., Kersters, K., De Vos, P., Logan, N.A., Ali, N., Berkeley, R.C.W., 1996. Reclassification of Paenibacillus (formerly Bacillus) pulvifaciens (Nakamura 1984) Ash et al. 1994, a later subjective synonym of Paenibacillus (formerly Bacillus) larvae (White 1906) Ash et al. 1994, as a subspecies of P. larvae, with emended descriptions of P. larvae as P. larvae subsp. larvae and P. larvae subsp. pulvifaciens. International journal of systematic and evolutionary microbiology 46, 270–279.

Higes, M., Martín-Hernández, R., Botías, C., Bailón, E.G., González-Porto, A.V., Barrios, L., Del Nozal, M.J., Bernal, J.L., Jiménez, J.J., Palencia, P.G., 2008. How natural infection by Nosema ceranae causes honeybee colony collapse. Environmental microbiology 10, 2659–2669.

Hitchcock, J., Stoner, A., Wilson, W., Menapace, D., 1979. Pathogenicity of Bacillus pulvifaciens to honey bee larvae of various ages (Hymenoptera: Apidae). Journal of the Kansas Entomological Society 238–246.

Hornitzky, M., Oldroyd, B.P., Somerville, D., 1996. Bacillus larvae carrier status of swarms and feral colonies of honeybees (Apis mellifera) in Australia. Australian veterinary journal 73, 116–117.

Huang, Z.Y., Bian, G., Xi, Z., Xie, X., 2019. Genes important for survival or reproduction in Varroa destructor identified by RNAi. Insect science 26, 68–75.

Hýbl, M., Bohatá, A., Rádsetoulalová, I., Kopecký, M., Hoštičková, I., Vaníčková, A., Mráz, P., 2021a. Evaluating the Efficacy of 30 Different Essential Oils against Varroa destructor and Honey Bee Workers (Apis mellifera). Insects 12, 1045.

Hybl, M., Mraz, P., Sipos, J., 2020. Diversity of bees (Apoidea) and their pesticide contamination in two different types of agricultural management, in: MendelNet. Brno, pp. 216–221.

Hýbl, M., Mráz, P., Šipoš, J., Hoštičková, I., Bohatá, A., Čurn, V., Kopec, T., 2021b. Polyphenols as Food Supplement Improved Food Consumption and Longevity of Honey Bees (Apis mellifera) Intoxicated by Pesticide Thiacloprid. Insects 12, 572.

Iwasa, T., Motoyama, N., Ambrose, J.T., Roe, R.M., 2004. Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, Apis mellifera. Crop protection 23, 371–378.

Jagua-Gualdrón, A., Peña-Latorre, J.A., Fernadez-Bernal, R.E., 2020. Apitherapy for osteoarthritis: perspectives from basic research. Complementary medicine research 27, 184–192.

Jarosch, A., Moritz, R.F., 2012. RNA interference in honeybees: off-target effects caused by dsRNA. Apidologie 43, 128–138.

Johnson, R.M., Dahlgren, L., Siegfried, B.D., Ellis, M.D., 2013. Effect of in-hive miticides on drone honey bee survival and sperm viability. Journal of Apicultural Research 52, 88–95.

Johnson, R.M., Pollock, H.S., Berenbaum, M.R., 2009. Synergistic interactions between in-hive miticides in Apis mellifera. Journal of economic entomology 102, 474–479.

Kablau, A., Berg, S., Härtel, S., Scheiner, R., 2019. Hyperthermia treatment can kill immature and adult Varroa destructor mites without reducing drone fertility. Apidologie 1–9.

Kablau, A., Berg, S., Rutschmann, B., Scheiner, R., 2020. Short-term hyperthermia at larval age reduces sucrose responsiveness of adult honeybees and can increase life span. Apidologie 51, 570–582.

Kamler, M., Nesvorna, M., Stara, J., Erban, T., Hubert, J., 2016. Comparison of tau-fluvalinate, acrinathrin, and amitraz effects on susceptible and resistant populations of Varroa destructor in a vial test. Experimental and applied acarology 69, 1–9.

Kang, Y., Blanco, K., Davis, T., Wang, Y., DeGrandi-Hoffman, G., 2016. Disease dynamics of honeybees with Varroa destructor as parasite and virus vector. Mathematical biosciences 275, 71–92.

Kanga, L.H.B., Jones, W.A., James, R.R., 2003. Field trials using the fungal pathogen, Metarhizium anisopliae (Deuteromycetes: Hyphomycetes) to control the ectoparasitic mite, Varroa destructor (Acari: Varroidae) in honey bee, Apis mellifera (Hymenoptera: Apidae) colonies. Journal of economic entomology 96, 1091–1099.

Katznelson, H., 1950. Bacillus pulvifaciens (n. sp.), an organism associated with powdery scale of honeybee larvae. Journal of Bacteriology 59, 153–155.

Kim, S.H., Mondet, F., Herve, M., Mercer, A., 2018. Honey bees performing varroa sensitive hygiene remove the most mite-compromised bees from highly infested patches of brood. Apidologie 49, 335–345.

Kirrane, M.J., De Guzman, L.I., Whelan, P.M., Frake, A.M., Rinderer, T.E., 2018. Evaluations of the removal of Varroa destructor in Russian honey bee colonies that display different levels of Varroa sensitive hygienic activities. Journal of Insect Behavior 31, 283–297.

Klein, A.-M., Vaissiere, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C., Tscharntke, T., 2007. Importance of pollinators in changing landscapes for world crops. Proceedings of the royal society B: biological sciences 274, 303–313.

Kohno, H., Suenami, S., Takeuchi, H., Sasaki, T., Kubo, T., 2016. Production of knockout mutants by CRISPR/Cas9 in the european honeybee, Apis mellifera L. Zoological science 33, 505–512.

Kong, H.G., Kim, H.H., Chung, J., Jun, J., Lee, S., Kim, H.-M., Jeon, S., Park, S.G., Bhak, J., Ryu, C.-M., 2019. The Galleria mellonella hologenome supports microbiota-independent metabolism of long-chain hydrocarbon beeswax. Cell reports 26, 2451–2464.

Kralj, J., Fuchs, S., 2006. Parasitic Varroa destructor mites influence flight duration and homing ability of infested Apis mellifera foragers. Apidologie 37, 577–587.

Kulhanek, K., Steinhauer, N., Rennich, K., Caron, D.M., Sagili, R.R., Pettis, J.S., Ellis, J.D., Wilson, M.E., Wilkes, J.T., Tarpy, D.R., 2017. A national survey of managed honey bee 2015–2016 annual colony losses in the USA. Journal of Apicultural Research 56, 328–340.

Kuzyšinová, K., Mudroňová, D., Toporčák, J., Molnár, L., Javorský, P., 2016. The use of probiotics, essential oils and fatty acids in the control of American foulbrood and other bee diseases. Journal of Apicultural Research 55, 386–395.

Kwong, W.K., Mancenido, A.L., Moran, N.A., 2017. Immune system stimulation by the native gut microbiota of honey bees. Royal Society open science 4, 170003.

Larsen, A., Reynaldi, F.J., Guzmán-Novoa, E., 2019. Fundaments of the honey bee (Apis mellifera) immune system. Review. Revista mexicana de ciencias pecuarias 10, 705–728.

Le Conte, Y., De Vaublanc, G., Crauser, D., Jeanne, F., Rousselle, J.-C., Bécard, J.-M., 2007. Honey bee colonies that have survived Varroa destructor. Apidologie 38, 566–572.

Le Conte, Y., Navajas, M., 2008. Climate change: impact on honey bee populations and diseases. Revue Scientifique et Technique-Office International des Epizooties 27, 499–510.

Lecocq, A., Foley, K., Jensen, A.B., 2018. Drone brood production in Danish apiaries and its potential for human consumption. Journal of Apicultural Research 57, 331–336.

Leonard, S.P., Powell, J.E., Perutka, J., Geng, P., Heckmann, L.C., Horak, R.D., Davies, B.W., Ellington, A.D., Barrick, J.E., Moran, N.A., 2020. Engineered symbionts activate honey bee immunity and limit pathogens. Science 367, 573–576.

Li, X., He, T., Wang, X., Shen, M., Yan, X., Fan, S., Wang, L., Wang, X., Xu, X., Sui, H., She, G., 2019. Traditional uses, chemical constituents and biological activities of plants from the genus Thymus. Chemistry & biodiversity 19, e1900254.

Li, Z., You, X., Wang, L., Yan, Z., Zhou, Z., 2018. Spore morphology and ultrastructure of an Ascosphaera apis strain from the honeybees (Apis mellifera) in southwest China. Mycologia 110, 325–338.

Lin, Z., Qin, Y., Page, P., Wang, S., Li, L., Wen, Z., Hu, F., Neumann, P., Zheng, H., Dietemann, V., 2018. Reproduction of parasitic mites Varroa destructor in original and new honeybee hosts. Ecology and evolution 8, 2135–2145.

Lippert, C., Feuerbacher, A., Narjes, M., 2021. Revisiting the economic valuation of agricultural losses due to large-scale changes in pollinator populations. Ecological Economics 180, 106860.

Llorens-Picher, M., Higes, M., Martín-Hernández, R., De la Rúa, P., Muñoz, I., Aidoo, K., Bempong, E.O., Polkuraf, F., Meana, A., 2017. Honey bee pathogens in Ghana and the presence of contaminated beeswax. Apidologie 48, 732–742.

Locke, B., 2016. Natural Varroa mite-surviving Apis mellifera honeybee populations. Apidologie 47, 467–482.

Locke, B., Forsgren, E., Fries, I., de Miranda, J.R., 2012. Acaricide treatment affects viral dynamics in Varroa destructor-infested honey bee colonies via both host physiology and mite control. Applied and environmental Microbiology 78, 227–235.

Loucif-Ayad, W., Aribi, N., Smagghe, G., Soltani, N., 2010. A scientific note on the impact of acaracides on the nutritional biochemistry of Apis mellifera intermissa (Hymenoptera: Apidae). Apidologie 41, 135–137.

Loucif-Ayad, W., Aribi, N., Soltani, N., 2008. Evaluation of secondary effects of some acaricides on Apis mellifera intermissa (Hymenoptera, Apidae): acetylcholinesterase and glutathione S-transferase activities. Eur. J. Sci. Res 21, 642–649.

Lourenço, A.P., Guidugli-Lazzarini, K.R., Freitas, F.C., Bitondi, M.M., Simões, Z.L., 2013. Bacterial infection activates the immune system response and dysregulates microRNA expression in honey bees. Insect biochemistry and molecular biology 43, 474–482.

Lu, J., Cokcetin, N.N., Burke, C.M., Turnbull, L., Liu, M., Carter, D.A., Whitchurch, C.B., Harry, E.J., 2019. Honey can inhibit and eliminate biofilms produced by Pseudomonas aeruginosa. Scientific reports 9, 1–13.

Maggi, M., Tourn, E., Negri, P., Szawarski, N., Marconi, A., Gallez, L., Medici, S., Ruffinengo, S., Brasesco, C., De Feudis, L., 2016. A new formulation of oxalic acid for Varroa destructor control applied in Apis mellifera colonies in the presence of brood. Apidologie 47, 596–605.

Mallon, E.B., Brockmann, A., Schmid-Hempel, P., 2003. Immune response inhibits associative learning in insects. Proceedings of the Royal Society of London. Series B: Biological Sciences 270, 2471–2473.

Mannino, M.C., Huarte-Bonnet, C., Davyt-Colo, B., Pedrini, N., 2019. Is the insect cuticle the only entry gate for fungal infection? Insights into alternative modes of action of entomopathogenic fungi. Journal of fungi 5, 33.

Marringa, W.J., Krueger, M.J., Burritt, N.L., Burritt, J.B., 2014. Honey bee hemocyte profiling by flow cytometry. PloS one 9, e108486.

Martin, S.J., Hawkins, G.P., Brettell, L.E., Reece, N., Correia-Oliveira, M.E., Allsopp, M.H., 2019. Varroa destructor reproduction and cell re-capping in mite-resistant Apis mellifera populations. Apidologie 1–13.

Masotti, V., Juteau, F., Bessière, J.M., Viano, J., 2003. Seasonal and phenological variations of the essential oil from the narrow endemic species Artemisia molinieri and its biological activities. Journal of agricultural and food chemistry 51, 7115–7121.

McAfee, A., Chapman, A., Iovinella, I., Gallagher-Kurtzke, Y., Collins, T.F., Higo, H., Madilao, L.L., Pelosi, P., Foster, L.J., 2018. A death pheromone, oleic acid, triggers hygienic behavior in honey bees (Apis mellifera L.). Scientific reports 8, 1–13.

McMahon, D.P., Natsopoulou, M.E., Doublet, V., Fürst, M., Weging, S., Brown, M.J., Gogol-Döring, A., Paxton, R.J., 2016. Elevated virulence of an emerging viral genotype as a driver of honeybee loss. Proceedings of the Royal Society B: Biological Sciences 283, 20160811.

Medici, S.K., Castro, A., Sarlo, E.G., Marioli, J.M., Eguaras, M.J., 2012. The concentration effect of selected acaricides present in beeswax foundation on the survival of Apis mellifera colonies. Journal of Apicultural Research 51, 164–168.

Meikle, W.G., Mercadier, G., Holst, N., Girod, V., 2008. Impact of two treatments of a formulation of Beauveria bassiana (Deuteromycota: Hyphomycetes) conidia on Varroa mites (Acari: Varroidae) and on honeybee (Hymenoptera: Apidae) colony health, in: Diseases of Mites and Ticks. Springer, pp. 105–117.

Meikle, W.G., Sammataro, D., Neumann, P., Pflugfelder, J., 2012. Challenges for developing pathogen-based biopesticides against Varroa destructor (Mesostigmata: Varroidae). Apidologie 43, 501–514.

Messan, K., Rodriguez, M.M., Chen, J., DeGrandi-Hoffman, G., Kang, Y., 2021. Population dynamics of Varroa mite and honeybee: Effects of parasitism with age structure and seasonality. Ecological Modelling 440, 109359.

Milano, F., Donnarumma, L., 2017. Determination of essential oils residues on zucchini fruits by GC-MS. Natural product research 31, 976–979.

Morales, M.M., Ramos, M.J.G., Vázquez, P.P., Galiano, F.J.D., Valverde, M.G., López, V.G., Flores, J.M., Fernández-Alba, A.R., 2020. Distribution of chemical residues in the beehive compartments and their transfer to the honeybee brood. Science of The Total Environment 710, 136288.

Moret, Y., Schmid-Hempel, P., 2000. Survival for immunity: the price of immune system activation for bumblebee workers. Science 290, 1166–1168.

Morfin, N., Goodwin, P.H., Guzman-Novoa, E., 2020. The Combined Effects of Varroa destructor Parasitism and Exposure to Neonicotinoids Affects Honey Bee (Apis mellifera L.) Memory and Gene Expression. Biology 9, 237.

Motta, E.V., Raymann, K., Moran, N.A., 2018. Glyphosate perturbs the gut microbiota of honey bees. Proceedings of the National Academy of Sciences 115, 10305–10310.

Mraz, P., Bohata, A., Hostickova, I., Kopecky, M., Zabka, M., Hybl, M., Curn, V., 2019. Inhibitory effect of selected botanical compounds on the honey bee fungal pathogen Ascosphaera apis. Proceedings of the MendelNet, Brno, Czech Republic 6–7.

Mráz, P., Hýbl, M., Kopecký, M., Bohatá, A., Hoštičková, I., Šipoš, J., Vočadlová, K., Čurn, V., 2021a. Screening of Honey Bee Pathogens in the Czech Republic and Their Prevalence in Various Habitats. Insects 12, 1051.

Mráz, P., Hýbl, M., Kopecký, M., Bohatá, A., Konopická, J., Hoštičková, I., Konvalina, P., Šipoš, J., Rost, M., Čurn, V., 2021b. The Effect of Artificial Media and Temperature on the Growth and Development of the Honey Bee Brood Pathogen Ascosphaera apis. Biology 10, 431.

Mraz, P., Hybl, M., Kopecky, M., Sipos, J., Ryba, S., Curn, V., 2020. Effect of the digestive process of the Greater wax moth (Galleria mellonella) on the causative agents of American Foulbrood (Paenibacillus larvae), in: MendelNet. Brno, pp. 413–418.

Muijres, F.T., Van Dooremalen, C., Lankheet, M., Lugt, H., De Vries, L.J., Van Langevelde, F., 2020. Varroa destructor infestation impairs the improvement of landing performance in foraging honeybees. Royal Society open science 7, 201222.

Munawar, M.S., Raja, S., Waghchoure, E.S., Barkat, M., 2010. Controlling American Foulbrood in honeybees by shook swarm method. Pakistan Journal of Agricultural Research 23.

Natsopoulou, M.E., McMahon, D.P., Doublet, V., Frey, E., Rosenkranz, P., Paxton, R.J., 2017. The virulent, emerging genotype B of Deformed wing virus is closely linked to overwinter honeybee worker loss. Scientific Reports 7, 1–9.

Negri, P., Maggi, M., Correa-Aragunde, N., Brasesco, C., Eguaras, M., Lamattina, L., 2013. Nitric oxide participates at the first steps of Apis mellifera cellular immune activation in response to non-self recognition. Apidologie 44, 575–585.

Negri, P., Maggi, M., Massazza, D., Correa-Aragunde, N., Eguaras, M., Lamattina, L., 2012. Nitric oxide stimulates melanin production during immune response in Apis mellifera. Biocell 36, 68.

Neuendorf, S., Hedtke, K., Tangen, G., Genersch, E., 2004. Biochemical characterization of different genotypes of Paenibacillus larvae subsp. larvae, a honey bee bacterial pathogen. Microbiology 150, 2381–2390.

Nolan, M.P., Delaplane, K.S., 2017. Distance between honey bee Apis mellifera colonies regulates populations of Varroa destructor at a landscape scale. Apidologie 48, 8–16.

Nolan, V.C., Harrison, J., Cox, J.A., 2019. Dissecting the antimicrobial composition of honey. Antibiotics 8, 251.

Nunes, F.M., Aleixo, A.C., Barchuk, A.R., Bomtorin, A.D., Grozinger, C.M., Simões, Z.L., 2013. Non-target effects of green fluorescent protein (GFP)-derived double-stranded RNA (dsRNA-GFP) used in honey bee RNA interference (RNAi) assays. Insects 4, 90–103.

Ongus, J.R., Peters, D., Bonmatin, J.-M., Bengsch, E., Vlak, J.M., van Oers, M.M., 2004. Complete sequence of a picorna-like virus of the genus Iflavirus replicating in the mite Varroa destructor. Journal of General Virology 85, 3747–3755.

Orantes-Bermejo, F.J., Pajuelo, A.G., Megías, M.M., Fernández-Píñar, C.T., 2010. Pesticide residues in beeswax and beebread samples collected from honey bee colonies (Apis mellifera L.) in Spain. Possible implications for bee losses. Journal of Apicultural Research 49, 243–250.

Page, P., Lin, Z., Buawangpong, N., Zheng, H., Hu, F., Neumann, P., Chantawannakul, P., Dietemann, V., 2016. Social apoptosis in honey bee superorganisms. Scientific Reports 6, 1–6.

Panziera, D., van Langevelde, F., Blacquière, T., 2017. Varroa sensitive hygiene contributes to naturally selected varroa resistance in honey bees. Journal of Apicultural Research 56, 635–642.

Papežíková, I., Palíková, M., Kremserová, S., Zachová, A., Peterová, H., Babák, V., Navrátil, S., 2017. Effect of oxalic acid on the mite Varroa destructor and its host the honey bee Apis mellifera. Journal of Apicultural Research 56, 400–408.

Partap, U., Ya, T., 2012. The human pollinators of fruit crops in Maoxian County, Sichuan, China. Mountain Research and Development 32, 176–186.

Pavela, R., Benelli, G., 2016. Essential oils as ecofriendly biopesticides? Challenges and constraints. Trends in plant science 21, 1000–1007.

Peck, D.T., 2021. The Parasitic Mite Varroa destructor: History, Biology, Monitoring, and Management. Honey Bee Medicine for the Veterinary Practitioner 235–251.

Peck, D.T., Seeley, T.D., 2019. Mite bombs or robber lures? The roles of drifting and robbing in Varroa destructor transmission from collapsing honey bee colonies to their neighbors. PloS one 14, e0218392.

Perricone, M., Arace, E., Corbo, M.R., Sinigaglia, M., Bevilacqua, A., 2015. Bioactivity of essential oils: a review on their interaction with food components. Frontiers in microbiology 6, 76.

Pettis, J., Wilson, W., Shimanuki, H., Teel, P., 1991. Fluvalinate treatment of queen and worker honey bees (Apis mellifera L) and effects on subsequent mortality, queen acceptance and supersedure. Apidologie 22, 1–7.

Pettis, J.S., Collins, A.M., Wilbanks, R., Feldlaufer, M.F., 2004. Effects of coumaphos on queen rearing in the honey bee, Apis mellifera. Apidologie 35, 605–610.

Pietropaoli, M., Formato, G., 2019. Acaricide efficacy and honey bee toxicity of three new formic acid-based products to control Varroa destructor. Journal of Apicultural Research 58, 824–830.

Pietropaoli, M., Formato, G., 2018. Liquid formic acid 60% to control varroa mites (Varroa destructor) in honey bee colonies (Apis mellifera): protocol evaluation. Journal of Apicultural Research 57, 300–307.

Poppinga, L., Genersch, E., 2015. Molecular pathogenesis of American Foulbrood: how Paenibacillus larvae kills honey bee larvae. Current opinion in insect science 10, 29–36.

Porrini, C., Sabatini, A.G., Girotti, S., Ghini, S., Medrzycki, P., Grillenzoni, F., Bortolotti, L., Gattavecchia, E., Celli, G., 2003. Honey bees and bee products as monitors of the environmental contamination. Apiacta 38, 63–70.

Posada-Florez, F., Ryabov, E.V., Heerman, M.C., Chen, Y., Evans, J.D., Sonenshine, D.E., Cook, S.C., 2020. Varroa destructor mites vector and transmit pathogenic honey bee viruses acquired from an artificial diet. PloS one 15, e0242688.

Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., Kunin, W.E., 2010. Global pollinator declines: trends, impacts and drivers. Trends in ecology & evolution 25, 345–353.

Potts, S.G., Neumann, P., Vaissière, B., Vereecken, N.J., 2018. Robotic bees for crop pollination: Why drones cannot replace biodiversity. Science of the total environment 642, 665–667.

Pritchard, D.J., 2016. Grooming by honey bees as a component of varroa resistant behavior. Journal of Apicultural Research 55, 38–48.

Ramsey, S.D., Ochoa, R., Bauchan, G., Gulbronson, C., Mowery, J.D., Cohen, A., Lim, D., Joklik, J., Cicero, J.M., Ellis, J.D., 2019. Varroa destructor feeds primarily on honey bee fat body tissue and not hemolymph. Proceedings of the National Academy of Sciences 116, 1792–1801.

Ramzi, H., Ismaili, M.R., Aberchane, M., Zaanoun, S., 2017. Chemical characterization and acaricidal activity of Thymus satureioides C. & B. and Origanum elongatum E. & M.(Lamiaceae) essential oils against Varroa destructor Anderson & Trueman (Acari: Varroidae). Industrial Crops and Products 108, 201–207.

Rangel, J., Tarpy, D.R., 2015. The combined effects of miticides on the mating health of honey bee (Apis mellifera L.) queens. Journal of Apicultural Research 54, 275–283.

Rangel, J., Ward, L., 2018. Evaluation of the predatory mite Stratiolaelaps scimitus for the biological control of the honey bee ectoparasitic mite Varroa destructor. Journal of Apicultural Research 57, 425–432.

Rattan, R.S., 2010. Mechanism of action of insecticidal secondary metabolites of plant origin. Crop protection 29, 913–920.

Rauch, S., Ashiralieva, A., Hedtke, K., Genersch, E., 2009. Negative correlation between individual-insect-level virulence and colony-level virulence of Paenibacillus larvae, the etiological agent of American foulbrood of honeybees. Applied and Environmental Microbiology 75, 3344–3347.

Raymann, K., Shaffer, Z., Moran, N.A., 2017. Antibiotic exposure perturbs the gut microbiota and elevates mortality in honeybees. PLoS biology 15, e2001861.

Read, S., Howlett, B.G., Donovan, B.J., Nelson, W.R., Van Toor, R.F., 2014. Culturing chelifers (Pseudoscorpions) that consume Varroa mites. Journal of applied entomology 138, 260–266.

Regnault-Roger, C., Vincent, C., Arnason, J.T., 2012. Essential oils in insect control: low-risk products in a high-stakes world. Annual review of entomology 57, 405–424.

Reilly, J., Artz, D., Biddinger, D., Bobiwash, K., Boyle, N., Brittain, C., Brokaw, J., Campbell, J., Daniels, J., Elle, E., 2020. Crop production in the USA is frequently limited by a lack of pollinators. Proceedings of the Royal Society B 287, 20200922.

Riessberger-Galle, U., Von Der Ohe, W., Crailsheim, K., 2001. Adult honeybee's resistance against Paenibacillus larvae larvae, the causative agent of the American foulbrood. Journal of invertebrate pathology 77, 231–236.

Rinkevich, F.D., 2020. Detection of amitraz resistance and reduced treatment efficacy in the Varroa Mite, Varroa destructor, within commercial beekeeping operations. PloS one 15, e0227264.

Rios, J.-L., Recio, M.C., 2005. Medicinal plants and antimicrobial activity. Journal of ethnopharmacology 100, 80–84.

Romo-Chacón, A., Martínez-Contreras, L.J., Molina-Corral, F.J., Acosta-Muñiz, C.H., Ríos-Velasco, C., de León-Door, A.P., Rivera, R., 2016. Evaluation of Oregano (Lippia berlandieri) Essential Oil and Entomopathogenic Fungi for Varroa destructor1 Control in Colonies of Honey Bee, Apis mellifera2. Southwestern Entomologist 41, 971–982.

Rosenkranz, P., Aumeier, P., Ziegelmann, B., 2010. Biology and control of Varroa destructor. Journal of invertebrate pathology 103, S96–S119.

Roth, A., Vleurinck, C., Netschitailo, O., Bauer, V., Otte, M., Kaftanoglu, O., Page, R.E., Beye, M., 2019. A genetic switch for worker nutrition-mediated traits in honeybees. PLoS biology 17, e3000171.

Roth, M.A., Wilson, J.M., Tignor, K.R., Gross, A.D., 2020. Biology and management of Varroa destructor (Mesostigmata: Varroidae) in Apis mellifera (Hymenoptera: Apidae) colonies. Journal of Integrated Pest Management 11, 1.

Rueppell, O., Hayworth, M.K., Ross, N., 2010. Altruistic self-removal of health-compromised honey bee workers from their hive. Journal of evolutionary biology 23, 1538–1546.

Ruffinengo, S.R., Maggi, M.D., Marcangeli, J.A., Eguaras, M.J., Principal, J., Barrios, C., de Piano, F.G., Giullia, M., 2014. Integrated Pest Management to control Varroa destructor and its implications to Apis mellifera colonies.

Ruoff, K., Bogdanov, S., 2004. Authenticity of honey and other bee products. Apiacta 38, 317–327.

Sabahi, Q., Morfin, N., Emsen, B., Gashout, H.A., Kelly, P.G., Otto, S., Merrill, A.R., Guzman-Novoa, E., 2020. Evaluation of Dry and Wet Formulations of Oxalic Acid, Thymol, and Oregano Oil for Varroa Mite (Acari: Varroidae) Control in Honey Bee (Hymenoptera: Apidae) Colonies. Journal of Economic Entomology 113, 2588–2594.

Saccà, M.L., Lodesani, M., 2020. Isolation of bacterial microbiota associated to honey bees and evaluation of potential biocontrol agents of Varroa destructor. Beneficial Microbes 11, 641–654.

Sahlan, M., Karwita, S., Gozan, M., Hermansyah, H., Yohda, M., Yoo, Y.J., Pratami, D.K., 2019. Identification and classification of honey's authenticity by attenuated total reflectance Fourier-transform infrared spectroscopy and chemometric method. Veterinary world 12, 1304.

Salyers, A.A., Whitt, D.D., 2002. A molecular approach. Bacterial pathogenesis.

Satta, A., Floris, I., Eguaras, M., Cabras, P., Garau, V.L., Melis, M., 2005. Formic acid-based treatments for control of Varroa destructor in a Mediterranean area. Journal of economic entomology 98, 267–273.

Schmid, M.R., Brockmann, A., Pirk, C.W., Stanley, D.W., Tautz, J., 2008. Adult honeybees (Apis mellifera L.) abandon hemocytic, but not phenoloxidase-based immunity. Journal of insect physiology 54, 439–444.

Schneider, S., Eisenhardt, D., Rademacher, E., 2012. Sublethal effects of oxalic acid on Apis mellifera (Hymenoptera: Apidae): changes in behaviour and longevity. Apidologie 43, 218–225.

Schöning, C., Gisder, S., Geiselhardt, S., Kretschmann, I., Bienefeld, K., Hilker, M., Genersch, E., 2012. Evidence for damage-dependent hygienic behaviour towards Varroa destructor-parasitised brood in the western honey bee, Apis mellifera. Journal of Experimental Biology 215, 264–271.

Schwartz, K.R., Minor, H., Magro, C., McConnell, J., Capani, J., Griffin, J., Doebel, H., 2020. The neonicotinoid imidacloprid alone alters the cognitive behavior in Apis mellifera L. and the combined exposure of imidacloprid and Varroa destructor mites synergistically contributes to trial attrition. Journal of Apicultural Research 60, 431–438.

Seeley, T.D., Griffin, S.R., 2011. Small-cell comb does not control Varroa mites in colonies of honeybees of European origin. Apidologie 42, 526–532.

Seeley, T.D., Tarpy, D.R., 2007. Queen promiscuity lowers disease within honeybee colonies. Proceedings of the Royal Society B: Biological Sciences 274, 67–72.

Serra Bonvehí, J., Ventura Coll, F., Ruiz Martínez, J.A., 2016. Residues of essential oils in honey after treatments to control Varroa destructor. Journal of essential oil research 28, 22–28.

Shakib, V., Mehdi, E.S., 2017. Infestation levels of the mite (Varroa destructor) between two honeybee races Apis mellifera meda (Indigenous) and Apis mellifera carnica (Imported) in Savojbolagh regions of Alborz province in Iran.

Shaw, K.E., Davidson, G., Clark, S.J., Ball, B.V., Pell, J.K., Chandler, D., Sunderland, K.D., 2002. Laboratory bioassays to assess the pathogenicity of mitosporic fungi to Varroa destructor (Acari: Mesostigmata), an ectoparasitic mite of the honeybee, Apis mellifera. Biological Control 24, 266–276.

Sidor, E., Dżugan, M., 2020. Drone Brood Homogenate as Natural Remedy for Treating Health Care Problem: A Scientific and Practical Approach. Molecules 25, 5699.

Silici, S., 2019. Chemical Content and Bioactive Properties of Drone Larvae (Apilarnil). Mellifera 19, 14–22.

Simone, M., Evans, J.D., Spivak, M., 2009. Resin collection and social immunity in honey bees. Evolution: International Journal of Organic Evolution 63, 3016–3022.

Sinia, A., Guzman-Novoa, E., 2018. Evaluation of the entomopathogenic fungi Beauveria bassiana GHA and Metarhizium anisopliae UAMH 9198 alone or in combination with thymol for the control of Varroa destructor in honey bee (Apis mellifera) colonies. Journal of Apicultural Research 57, 308–316.

Smart, M.D., Otto, C.R., Lundgren, J.G., 2019. Nutritional status of honey bee (Apis mellifera L.) workers across an agricultural land-use gradient. Scientific reports 9, 1–10.

Spiltoir, C.F., 1955. Life cycle of Ascosphaera apis (Pericystis apis). American Journal of Botany 501–508.

Spivak, M., Reuter, G.S., 2001. Resistance to American foulbrood disease by honey bee colonies Apis mellifera bred for hygienic behavior. Apidologie 32, 555–565.

Spivak, M., Reuter, G.S., 1998. Performance of hygienic honey bee colonies in a commercial apiary. Apidologie 29, 291–302.

Stara, J., Pekar, S., Nesvorna, M., Kamler, M., Doskocil, I., Hubert, J., 2019. Spatio-temporal dynamics of Varroa destructor resistance to tau-fluvalinate in Czechia, associated with L925V sodium channel point mutation. Pest management science 75, 1287–1294.

Starks, P.T., Blackie, C.A., Seeley, T.D., 2000. Fever in honeybee colonies. Naturwissenschaften 87, 229–231.

Stevenson, P.C., 2020. For antagonists and mutualists: the paradox of insect toxic secondary metabolites in nectar and pollen. Phytochemistry Reviews 19, 603–614.

Stroeymeyt, N., Casillas-Pérez, B., Cremer, S., 2014. Organisational immunity in social insects. Current Opinion in Insect Science 5, 1–15.

Svečnjak, L., Jović, O., Prđun, S., Rogina, J., Marijanović, Z., Car, J., Matošević, M., Jerković, I., 2019. Influence of beeswax adulteration with paraffin on the composition and quality of honey determined by physico-chemical analyses, 1H NMR, FTIR-ATR and HS-SPME/GC–MS. Food chemistry 291, 187–198.

Tak, J.H., Isman, M.B., 2017. Penetration-enhancement underlies synergy of plant essential oil terpenoids as insecticides in the cabbage looper, Trichoplusia ni. Scientific reports 7, 1–11.

Taning, C.N.T., Van Eynde, B., Yu, N., Ma, S., Smagghe, G., 2017. CRISPR/Cas9 in insects: Applications, best practices and biosafety concerns. Journal of insect physiology 98, 245–257.

Tarpy, D.R., 2003. Genetic diversity within honeybee colonies prevents severe infections and promotes colony growth. Proceedings of the Royal Society of London. Series B: Biological Sciences 270, 99–103.

Tewarson, N., 1982. Resorption of undigested proteins and their incorporation into the eggs of Varroa jacobsoni [honey-bees, Apis mellifera]. Apidologie (Germany, FR).

Thompson, H.M., Fryday, S.L., Harkin, S., Milner, S., 2014. Potential impacts of synergism in honeybees (Apis mellifera) of exposure to neonicotinoids and sprayed fungicides in crops. Apidologie 45, 545–553.

Tihelka, E., 2018. Effects of synthetic and organic acaricides on honey bee health: a review. Slovenian Veterinary Research 55, 114–40.

Titěra, D., 2009. Mor včelího plodu. VÚVč.

Tock, M.L.A., Kamatou, G., Combrinck, S., Sandasi, M., Viljoen, A., 2020. A chemometric assessment of essential oil variation of three Salvia species indigenous to South Africa. Phytochemistry 172, 112249.

Toomemaa, K., Martin, A.-J., Williams, I.H., 2010. The effect of different concentrations of oxalic acid in aqueous and sucrose solution on Varroa mites and honey bees. Apidologie 41, 643–653.

Toufailia, H.M.A., Amiri, E., Scandian, L., Kryger, P., Ratnieks, F.L., 2014. Towards integrated control of varroa: effect of variation in hygienic behaviour among honey bee colonies on mite population increase and deformed wing virus incidence. Journal of Apicultural Research 53, 555–562.

Traynor, K.S., Mondet, F., de Miranda, J.R., Techer, M., Kowallik, V., Oddie, M.A., Chantawannakul, P., McAfee, A., 2020. Varroa destructor: A complex parasite, crippling honey bees worldwide. Trends in Parasitology 36, 592–606.

Traynor, K.S., Pettis, J.S., Tarpy, D.R., Mullin, C.A., Frazier, J.L., Frazier, M., 2016. In-hive Pesticide Exposome: Assessing risks to migratory honey bees from in-hive pesticide contamination in the Eastern United States. Scientific reports 6, 1–16.

Tsakas, S., Marmaras, V., 2010. Insect immunity and its signalling: an overview. Invertebrate Survival Journal 7, 228–238.

Tsourkas, P.K., 2020. Paenibacillus larvae bacteriophages: Obscure past, promising future. Microbial genomics 6.

Ulmer, M., Smetana, S., Heinz, V., 2020. Utilizing honeybee drone brood as a protein source for food products: Life cycle assessment of apiculture in Germany. Resources, Conservation and Recycling 154, 104576.

Vázquez, P.P., Lozano, A., Uclés, S., Ramos, M.G., Fernández-Alba, A.R., 2015. A sensitive and efficient method for routine pesticide multiresidue analysis in bee pollen samples using gas and liquid chromatography coupled to tandem mass spectrometry. Journal of Chromatography A 1426, 161–173.

Vojvodic, S., Jensen, A.B., James, R.R., Boomsma, J.J., Eilenberg, J., 2011. Temperature dependent virulence of obligate and facultative fungal pathogens of honeybee brood. Veterinary Microbiology 149, 200–205.

von der Ohe, W., 2003. Control of American Foulbrood by using alternatively eradication method and artificial swarms. Apiacta 38, 137–139.

Wang, S., Lin, Z., Chen, G., Page, P., Hu, F., Niu, Q., Su, X., Chantawannakul, P., Neumann, P., Zheng, H., 2020. Reproduction of ectoparasitic mites in a coevolved system: Varroa spp.—Eastern honey bees, Apis cerana. Ecology and evolution 10, 14359–14371.

Wantuch, H.A., Tarpy, D.R., 2009. Removal of drone brood from Apis mellifera (Hymenoptera: Apidae) colonies to control Varroa destructor (Acari: Varroidae) and retain adult drones. Journal of economic entomology 102, 2033–2040.

Ward, K., Danka, R., Ward, R., 2008. Comparative performance of two mite-resistant stocks of honey bees (Hymenoptera: Apidae) in Alabama beekeeping operations. Journal of economic entomology 101, 654–659.

Wedenig, M., Riessberger-Gallé, U., Crailsheim, K., 2003. A substance in honey bee larvae inhibits the growth of Paenibacillus larvae larvae. Apidologie 34, 43–51.

Weick, J., Thorn, R.S., 2002. Effects of acute sublethal exposure to coumaphos or diazinon on acquisition and discrimination of odor stimuli in the honey bee (Hymenoptera: Apidae). Journal of economic entomology 95, 227–236.

White, G.F., 1906. Bacteria of the Apiary. Technical series (United States. Bureau of Entomology); no. 14.

Wilson, M.B., Pawlus, A.D., Brinkman, D., Gardner, G., Hegeman, A.D., Spivak, M., Cohen, J.D., 2017. 3-Acyl dihydroflavonols from poplar resins collected by honey bees are active against the bee pathogens Paenibacillus larvae and Ascosphaera apis. Phytochemistry 138, 83–92.

Wilson-Rich, N., Dres, S.T., Starks, P.T., 2008. The ontogeny of immunity: development of innate immune strength in the honey bee (Apis mellifera). Journal of insect physiology 54, 1392–1399.

Wu, J.Y., Anelli, C.M., Sheppard, W.S., 2011. Sub-lethal effects of pesticide residues in brood comb on worker honey bee (Apis mellifera) development and longevity. PloS one 6, e14720.

Wu, J.Y., Smart, M.D., Anelli, C.M., Sheppard, W.S., 2012. Honey bees (Apis mellifera) reared in brood combs containing high levels of pesticide residues exhibit increased susceptibility to Nosema (Microsporidia) infection. Journal of invertebrate pathology 109, 326–329.

Yang, D., Xu, X., Zhao, H., Yang, S., Wang, X., Zhao, D., Diao, Q., Hou, C., 2018. Diverse factors affecting efficiency of RNAi in honey bee viruses. Frontiers in genetics 9, 384.

Yue, D., Nordhoff, M., Wieler, L.H., Genersch, E., 2008. Fluorescence in situ hybridization (FISH) analysis of the interactions between honeybee larvae and Paenibacillus larvae, the causative agent of American foulbrood of honeybees (Apis mellifera). Environmental microbiology 10, 1612–1620.

Zaghloul, O., Mourad, A., El Kady, M.B., Nemat, F., Morsy, M., 2005. Assessment of losses in honey yield due to the chalkbrood disease, with reference to the determination of its economic injury levels in Egypt. Communications in agricultural and applied biological sciences 70, 703–714.

Zhang, Q., Ongus, J.R., Boot, W.J., Calis, J., Bonmatin, J., Bengsch, E., Peters, D., 2007. Detection and localisation of picorna-like virus particles in tissues of Varroa destructor, an ectoparasite of the honey bee, Apis mellifera. Journal of invertebrate pathology 96, 97–105.

Zhang, Y., Han, R., 2018. A saliva protein of Varroa mites contributes to the toxicity toward Apis cerana and the DWV elevation in A. mellifera. Scientific reports 8, 1–9.

Zheng, H., Powell, J.E., Steele, M.I., Dietrich, C., Moran, N.A., 2017. Honeybee gut microbiota promotes host weight gain via bacterial metabolism and hormonal signaling. Proceedings of the National Academy of Sciences 114, 4775–4780.

8 Seznam vlastních publikovaných prací

8.1 Publikované práce v impaktovaných časopisech

Kopecký, M., Kolář, L., Konvalina, P., Strunecký, O., Teodorescu, F., **Mráz, P.**, Peterka, J., Váchalová, R., Bernas, J., Bartoš, P., 2020. Modified Biochar—A Tool for Wastewater Treatment. Energies 13, 5270.

Mráz, P., Hýbl, M., Kopecký, M., Bohatá, A., Konopická, J., Hoštičková, I., Konvalina, P., Šipoš, J., Rost, M., Čurn, V., 2021. The Effect of Artificial Media and Temperature on the Growth and Development of the Honey Bee Brood Pathogen *Ascosphaera apis*. Biology 10, 431.

Kopecký, M., **Mráz, P.**, Kolář, L., Váchalová, R., Bernas, J., Konvalina, P., Perná, K., Murindangabo, Y., Menšík, L., 2021. Effect of Fertilization on the Energy Profit of Tall Wheatgrass and Reed Canary Grass. Agronomy 11, 445.

Hýbl, M., **Mráz, P.**, Šipoš, J., Hoštičková, I., Bohatá, A., Čurn, V., Kopec, T., 2021. Polyphenols as Food Supplement Improved Food Consumption and Longevity of Honey Bees (*Apis mellifera*) Intoxicated by Pesticide Thiacloprid. Insects 12, 572.

Mráz, P., Hýbl, M., Kopecký, M., Bohatá, A., Hoštičková, I., Šipoš, J., Vočadlová, K., Čurn, V. 2021. Screening of honey bee pathogens in the Czech Republic and their prevalence in various habitats. Insects 12, 1051.

Hýbl, M., Bohatá, A., Rádsetoulalová, I., Jelínková, I., Vaníčková, A., **Mráz, P.** 2021. Evaluating the efficacy of 30 different essential oils against *Varroa destructo*r and honey bee workers (*Apis mellifera*). Insects 12, 1045.

Kopecký, M., Kolář, L., Váchalová, R., Konvalina, P., Batt, J., **Mráz, P.**, Menšík, L., Hoang, T.N., Dumbrovský, M. 2021. Black carbon and its effect on carbon sequestration in soil. Agronomy 11, 2261.

Kopecký, M., Kolář, L., Perná, K., Váchalová, R., **Mráz, P.**, Konvalina, K., Murindangabo, Y.G., Dumbrovksý, M. 2021. Fractionation of soil organic matter into labile and stable fractions. Agronomy 12, 73.

Hýbl, M. Šipoš, J., Krejčová, A., Sodomová, K., Polák, O., Koláčková, I., **Mráz, P.** 2022 Preference of pollinators over various forage mixtures and microelement treatments. Agronomy 12, 370.

8.2 Rukopisy prací v recenzním řízení v impaktovaných časopisech

Mráz, P., Žabka, M., Hýbl, M., Kopecký, M., Bohatá, A., Tomčala, A., Čurn, V. 2021. Antifungal activity of selected botanical compounds on *Ascosphaera apis*. Industrial Crops and Products.

Hýbl, M., **Mráz, P.**, Vládek, A, Přidal, A., Polák, O., Šipoš, J. 2021. Determination of main pollinators of haskap (*Lonicera caerulea*: Caprifoliaceae) and the effect of different controlled methods of pollination on fruit set. Agriculture, Ecosystems and Environment.

8.3 Konferenční příspěvky uvedené v databázi WoS

Mraz, P., Bohata, A., Hostickova, I., Kopecky, M., Zabka, M., Hybl, M., Curn, V., 2019. Inhibitory effect of selected botanical compounds on the honey bee fungal pathogen *Ascosphaera apis*. Proceedings of the MendelNet, Brno, Czech Republic 6–7.

Hybl, M., **Mraz, P**., Sipos, J., Pridal, A., 2019. Effects of phenolic bioactive substances on reducing mortality of bees (*Apis mellifera*) intoxicated by thiacloprid. Proceedings of the MendelNet, Brno, Czech Republic 6–7.

8.4 Recenzované práce

Mráz, P., Bohatá, A., Konopická, J., Čurn, V. 2018. Vliv živné půdy a teploty na radiální růst entomopatogenní houby *Ascosphaera apis* způsobující onemocnění včelího plodu. Úroda 66: 235-238.

Konopická, J., Sandala, D., Bohatá, A., **Mráz, P**. 2018. Teplotní profil růstu a produkce spor entomopatogenní houby *Metarhizium Brunneum*. Úroda 66: 209-212.

Mraz, P., Hybl, M., Kopecky, M., Sipos, J., Ryba, S., Curn, V., 2020. Effect of the digestive process of the Greater wax moth (*Galleria mellonella*) on the causative agents of American Foulbrood (*Paenibacillus larvae*), in: MendelNet. Brno, pp. 413–418.

Hybl, M., **Mraz, P**., Sipos, J., 2020. Diversity of bees (Apoidea) and their pesticide contamination in two different types of agricultural management, in: MendelNet. Brno, pp. 216–221.

8.5 Kapitola v knize

Tawaha, A.R.M., Kozuharova, E., Valchev, H., Hýbl, M., **Mráz, P**., Kovářová, D., Elfattah, M.S.A., Trioui, L., Al-Tawaha, A.R., et al. 2022. Climate Change and Ecosystems. Chapter 16. Pollinators Ecology and Management. Taylor & Francis, 336p, ISBN 9781032260686. In Press.

8.6 Certifikovaná metodika

Čurn, V., Hoštičková, I., **Mráz, P**., Vočadlová, K., Stehlíková, D., Beneš, K., Rost, M. 2021. Metodický postup detekce exprese specifických genů významných pro detoxifikaci látek u včel.